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THE BIFIDOGENICITY OF THE PREBIOTIC GALACTOLOGOSACCHARIDES

by

Lauren M.G. Davis

A THESIS

Presented to the Faculty of

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THE BIFIDOGENICITY OF THE PREBIOTIC GALACTOLOGOSACCHARIDES

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University of Nebraska, 2010

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The goal of this research was to determine the effect of different doses of galactooligosaccharide (GOS) on the fecal microbiota of healthy adults, with a focus on bifidobacteria. The study was designed as a single-blinded study, with eighteen subjects consuming GOS-containing chocolate chews at four increasing dosage levels; 0, 2.5, 5.0, and 10.0 g. Subjects consumed each dose for 3 weeks, with a two-week baseline period preceding the study and a two-week washout period at the end. Cultural methods were used for bifidobacteria, *Bacteroides*, enterobacteria, enterococci, lactobacilli, and total anaerobes; culture-independent methods included denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qRT-PCR) using *Bifidobacterium*-specific primers. All three methods revealed an increase in bifidobacteria populations, as the GOS dosage increased to 5 or 10 g. Enumeration of bifidobacteria by qRT-PCR showed a high inter-subject variation in bifidogenic effect and indicated a subset of 9 GOS responders among the eighteen subjects. There were no differences, however, in the initial levels of bifidobacteria between

the responding individuals and the non-responding individuals. In order to gain a community wide perspective of the impact of GOS on the fecal microbiota of the subjects, we then performed high throughput multiplex community sequencing of 16S rRNA tags. Multiplex sequencing of the 16s rRNA tags revealed that GOS induced significant compositional alterations in the fecal microbial populations by increasing the phyla Actinobacteria. The population shifts caused by consumption of 10 g of GOS were numerically substantial, leading for example, to a ten-fold increase in bifidobacteria in four subjects, enriching them to 18-33% off the fecal microbial community, and a five-fold increase in seven additional subjects. This increase in bifidobacteria abundance was generally at the expense of only one group of bacteria, namely the genus *Bacteroides*. Collectively, this study showed that a high purity GOS, administered in a confection product at doses of 5 g or higher, was bifidogenic, while a dose of 2.5 gram showed no significant effect. Our results also demonstrated that GOS is remarkable for its ability to enrich specifically for bifidobacteria in human fecal samples.

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“Without a struggle, there can be no progress.” *Fredrick Douglas*

Exciting, rewarding, impact-full, frustrating, growing, challenging, journey, struggle; these are just a few of the words that describe the past few years as I progressed toward obtaining my Masters Degree. As I look back at what I have faced, I am thankful for where those struggles have taken me and the support and guidance I gained along the way.

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“Family faces are magic mirrors. Looking at people who belong to us, we see the past, present, and future.”

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Preface

This thesis is composed of four chapters. Chapter 1 provides a review of the current literature on the effects of prebiotics on the human intestinal microbiota, with a special focus on galactooligosaccharides (GOS). Chapter 2 describes our published (Davis et al., 2010 *Int. J. Food Microbiol.* In press) results focusing on the impact that consecutive doses of the prebiotic galactooligosaccharides have on the intestinal microbiota of healthy adults. Chapter 3 describes additional results from that study, where we focus on community sequencing and the highly specific bifidogenic response GOS has on the human gastrointestinal tract. Finally, Chapter 4 provides a conclusion section that summarizes the major research findings presented within this thesis and the suggested areas for future research.

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Chapter 1

Prebiotic Activity of Galactooligosaccharides:

A Review of structure, function, and *in vivo* human studies

Introduction

Consumers have become increasingly aware of the importance of the human gastrointestinal microbiota in promoting health and well-being (48, 55). However, because the human intestine is a complex ecosystem in which thousands of different bacterial species reside, defining the microbial composition and establishing the function of this ecosystem represent a considerable challenge. Nonetheless, there is now substantial interest in understanding the role of the colonic microbiota and developing the means to manipulate the composition of the intestinal microbiota to enhance human health (30). In particular, the role of diet has been the subject of much of this interest, due to the potential impact specific dietary substances may have on the intestinal environment.

Without doubt, the group of dietary substances that have received the most attention for their ability to modulate the colonic microbiota are the prebiotics. Prebiotics are defined as a “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (52). Most of the compounds currently recognized as prebiotics are carbohydrates, either polysaccharides or oligosaccharides. Although many carbohydrates have been suggested to have prebiotic activity (31), the most well-studied include the fructans, inulin, and fructooligosaccharides (FOS), and the galactooligosaccharides (GOS). The latter are of particular interest due to their

similarity to human milk oligosaccharides. Moreover, the ability of GOS to enrich for bifidobacteria and/or lactobacilli in the human intestinal tract is now well-established. However, many questions remain regarding the amount of GOS necessary to generate bifidogenic (or lactobacilligenic) changes in the human intestinal tract, the means of delivery and stability of GOS in foods, and the effect of GOS on the overall composition of the microbiota.

In this review, the classification, structure, and means of production of prebiotics, and GOS in particular, will be described. An overview of intestinal microbial ecology will also be described, including how prebiotics and GOS influence the microbiota. Finally, published results using GOS in human feeding trials, including studies with infants, healthy adults, and elderly adults will be reviewed.

Classification and Properties of Prebiotics

The recent commercialization of non-digestible oligosaccharides as food ingredients has triggered a vast amount of research on their potential role in colonic health. Since these non-digestible oligosaccharides are not hydrolyzed by enzymes in the human small intestine, they reach the colon almost intact, and have coined the name “prebiotics” (3). Prebiotics were first defined as a ‘non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the

colon, and thus improves host health' (30). This prebiotic definition has since then been revised to 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host welling-being and health' (53). Many food oligosaccharides and polysaccharides have claimed to provide prebiotic activity (including dietary fiber), however, not all dietary carbohydrates are considered prebiotics. Those classified as prebiotics are short-chain oligosaccharides and the degree of polymerization of these oligosaccharides varies from 2 to 60. (16, 64; Table 1). Based on this and other potential claims to follow, there is a need to establish clear criteria for classifying a prebiotic. These classifications require that the ingredient demonstrates (i) resistance to hydrolysis and absorption in the upper part of the gastrointestinal tract; (ii) serve as a selective substrate for fermentation by one or a limited number of potentially beneficial bacteria in the colon, resulting in an increase in their growth and/or metabolic activity; (iii) alter the composition of the colonic microflora toward a healthier composition, and (iv) encourage effects that are beneficial to the host's health(24, 30, 70, 71). While the research on prebiotics is continuing to surge, most of the studies involving prebiotic oligosaccharides have been carried out using inulin and its fructooligosaccharide (FOS) derivatives, together with various forms of galactooligosaccharides (GOS). Specifically, inulin and FOS have been studied intensively *in vivo* over the past decade, while the studies associated with GOS are still young but hold potentially promising data for the prebiotic field.

Table 1. Carbohydrates used as prebiotics¹

Fructooligosaccharides (FOS)
 Galactooligosaccharides (GOS)
 Gentiooligosaccharides
 Inulin
 Isomaltooligosaccharides
 Lactulose
 Lactosucrose
 Soybean oligosaccharides
 Xylooligosaccharides

¹ Adapted from (67, 68)

Galactooligosaccharides (GOS)

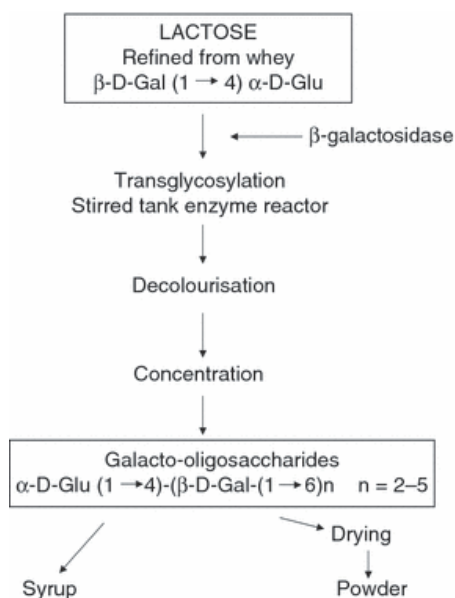
Human Milk Oligosaccharides – GOS. For the first few months of life, human milk is often the sole dietary source. Not only does it contain all the nutrients necessary for infants to thrive, but also nutrients that may provide health benefits beyond those of traditional nutrients, such as GOS. Human milk is considered one of the earliest sources of GOS with approximately 7% carbohydrates, 90% of which is lactose, and a variety of oligosaccharides based on lactose (21, 57). Oligosaccharides, after lactose and lipids, make up the third largest component in human milk (61), and are found at their highest levels in colostrum, where they can reach up to 24% of the total colostrum carbohydrates. In the first two months after birth, the concentrations of these oligosaccharides steadily decrease to between 19% and 15% (57). Human milk oligosaccharides, once ingested, can withstand the low pH in the gut (32) and resist degradation through enzymes from the pancreas and brush border membrane (20, 32). Once these human milk oligosaccharides reach the gastrointestinal tract, they have been shown to

establish a microbiota predominant in lactobacilli and bifidobacteria (45), which differs from that of infants fed on cow's milk (37, 51). Oligosaccharides in milk can reach as high as 8-12 g l⁻¹ (46, 47), which is 100 times greater than in cow's milk. The ability of breast-fed infants to utilize oligosaccharides in breast milk, including GOS which infants cannot digest in the upper gut, is thought to be the reason for the predominance of bifidobacteria within the gastrointestinal microflora (20, 37, 62). When human milk is not available for various reasons, incorporation of manufactured prebiotics such as GOS can be a useful addition to formulas so that functional characteristics associated with breast milk can be replicated, such as its bifidogenic effects (52). A mixture can be developed for formula that is based on the analysis of human milk and the high concentration of galactose. These formulas can later be fed to infants to obtain comparisons of gut microbiota and fecal fermentation product composition to determine the closeness of relation to that of breast-fed infants (see later).

Composition and properties of GOS. There are three main methods by which prebiotic oligosaccharides are produced: (i) plant extraction of natural oligosaccharides, (ii) monitored hydrolysis of natural polysaccharides, and (iii) using hydrolases and/or glycosyl transferases from microbial or plant sources for enzymatic synthesis (34, 49). The final structure composition of the prebiotic is dependent on the method in which the prebiotic is produced. Linkages that are recalcitrant to hydrolysis by human or microbial hydrolases, such as β -glycosidic linkages, are generally found in all prebiotics (72). GOS are produced from

lactose through the action of β -galactosidases, and, depending on the source of the β -galactosidase, different synthetic product mixtures are formed (19). In general, β -galactosidase is known as an enzyme that is able to catalyze the hydrolysis of lactose, while also being able to catalyze the transgalactosylation reaction needed to develop the GOS product (28, 65). It is thought that increased specificity of GOS can be obtained by using β -GOS, which is synthesized by bifidobacteria, utilizing their own β -galactosidase in the manufacturing of the product. GOS consists of chains of galactose molecules ending in a terminal glucose molecule, with a degree of polymerization (DP) from between 2 to 10 and various types of linkages. Production of GOS for commercial products utilizes whey-derived lactose as the main raw material (4, 102), however GOS can be produced from lactose in cow's milk. Since whey is produced in large amounts by the dairy industry as a by-product of cheese making, it becomes a more efficient way of reducing waste (52). When in syrup form, GOS is usually transparent and more viscous than high-fructose corn syrups (HFCS). When compared to sucrose, GOS has about one third of the sweetness (63), and shows a good moisture retention and high solubility (52).

Figure 1. Synthesis of galactooligosaccharides from lactose refined from whey¹.



¹Adapted from (52)

GOS Stability in Foods. As effective functional food ingredients, prebiotics must be chemically stable to food processing conditions. Many processing treatments involve low pH or heat, such as yogurt fermentation or pasteurization, which may hydrolyze the prebiotics to their respective monosaccharides. In addition, some prebiotic structures consist of reducing ends, which through Maillard reactions may interact with amino acids present in proteins. Hydrolyzation of prebiotics would not allow them to retain prebiotic activity *in vivo*, due to the released monosaccharides being absorbed in the intestinal tract. This would allow the prebiotics to be utilized by the general commensal microflora, instead of selecting for beneficial intestinal and/or prebiotic bacteria in the colon. Maillard reactions may also reduce the prebiotic activity and make them no longer available for metabolism by beneficial bacteria. The chemical

stability of GOS was reported in a study performed by Sako et al (1999). Using aqueous buffered solutions of Oligomate 55, stability was observed at 100°C and pH 2, 120°C and pH3, and at 160°C and neutral pH. When compared to sucrose at 10% concentration, GOS was found to be about three times more stable, as sucrose was degraded under many of the conditions tested. GOS was also found to be stable when tested in acidic conditions during long-term storage at room temperature. Due to their stability, GOS can be incorporated into a wide variety of foods (in some cases coupled with FOS) and are currently used in commercial commodities including infant formulas, dairy products, sauces, soups, breakfast cereals, beverages, snack bars, ice creams, bakery products, animal feeds, and as sugar replacements (103).

Health benefits linked to GOS. The human gastrointestinal tract, especially the colon, is a somewhat recently explored microbial ecosystem, offering a good opportunity for the development of dietary interventions targeting disease reduction risk and maintenance of good health. Since GOS has been shown to be fermented by the colonic bacterial flora, in particular bifidobacteria, studies investigating the potential bifidogenic relationship are ever growing. The bifidobacteria population is not only important for the eco-physiology of the colonic microbiota; they are also believed to have health benefits. Evidence has shown that bifidobacteria are the main species colonizing the infant gastrointestinal microbiota, which in turn has decreased the overall numbers of potentially pathogenic bacteria. However, with age, the number of bifidobacteria

begins to decrease and is slowly replaced by bacteria mainly associated within the phylum Firmicutes or Bacteroidetes. Due to this decrease, efforts to restore or increase this population through the use of prebiotics, especially GOS, has become of great interest. These organisms have been linked to increased resistance to infection and diarrheal disease (33, 74, 93, 101), stimulation of immune system activity (44, 77), as well as protection against cancer (69, 78). In animal models, some bifidobacteria manifest strong anti-mutagenic and anti-tumor properties that have prophylactic and therapeutic benefits (39). Along with potential immune benefits, GOS has been studied as a potential therapeutic agent for IBS and IBD patients, by fermenting and increasing bifidobacteria that demonstrate pro-inflammatory like benefits and improve abdominal symptoms (82). GOS has also been linked to potential laxative-like properties within elderly by relieving constipation, however, responses differ individually (89). Small human trials, with mixed effects, involving GOS and the potential to stimulate true calcium absorption in postmenopausal women have also been performed. An increase in calcium absorption in postmenopausal women was observed, however further research is required for any definitive conclusions to be drawn (97). GOS has been under investigation in the area of cardiovascular effects as well, and very few trials have used it as a prebiotic. In a study performed by van Dokkum and colleagues (1999), GOS did not seem to alter blood lipid concentration and glucose absorption; however it should not be excluded for people with elevated blood lipid concentration or diabetic patients, where it

potentially could be beneficial. Another area of interest, with respect to prebiotic benefits, is allergic disorders. The prevalence of allergic disorders in developed countries has been on a steady increase over the last few decades. A delayed maturation of the immune system has been associated with a higher risk of allergies in children, and it has been suggested that breast feeding reduces this incidence. Since breast feeding is associated with high levels of GOS, supplementation of GOS has been shown to induce a beneficial antibody profile in infants at risk for allergy, while leaving the response to vaccination intact (99).

Given that positive results have arisen from animal studies, it is important that systematic studies are continually instigated to test for continued GOS benefits in humans. Researchers are probably just beginning to touch the full potential of health benefits conferred by GOS and so far, the majority of investigations have focused on more obvious gastrointestinal-related diseases. Other areas of health, however, will undoubtedly expand on these potential benefits in the future.

The Human Intestinal Microbiota

This predominantly anaerobic microbiota is able to salvage energy for the host by fermenting undigested carbohydrates and proteins to short-chain fatty acids, which are then absorbed (15). The intestinal microbiota may also synthesize vitamins (18), protect against invasive species that are often pathogenic (10, 26), and possibly contribute to the economy of essential amino acids in humans (85, 94).

Breakdown of polysaccharides in the large intestine is a complex process involving various enzymes (poly-, oligo- and monosaccharidases) from many different species, and cross-feeding by the microflora (15). Evidently the effect of such a process on the growth of specific bacteria is difficult to predict in an ecosystem consisting of such a large number of physiologically and nutritionally diverse bacterial species. Not all intestinal bacteria are beneficial to health, and a long-established concept is that of beneficial and possibly harmful species. Generally beneficial genera include *Bifidobacterium* and *Lactobacillus*, both of which are saccharolytic, whereas some species such as *Clostridium perfringens* and *Escherichia coli* can be considered harmful (29, 86). The human intestinal microbiota is affected by many factors such as age, drug therapy, disease, diet, host physiology, peristalsis, local immunity, and *in situ* bacterial metabolism (9). However, diet is probably the most significant factor determining the type of gastrointestinal microflora that develops since foodstuffs provide the main nutrient sources for colonic bacteria.

GOS Human feeding trials

Infants and pregnant mothers. Born with an essentially sterile gastrointestinal tract, infants provide a unique opportunity to investigate the effects of prebiotics on the gastrointestinal microbiota. Immediately after birth, the colonization of the infant gastrointestinal tract starts (73) and is influenced by the mode of delivery (8, 35), the composition of the maternal microbiota (87), and the mode of feeding – ie, breast milk or infant formula (37, 84). Over a series of weeks and months,

infants become progressively colonized by different bacteria, which results in the establishment of an increasingly complex and stable microbiota. Since the development of the gastrointestinal microbiota is profoundly influenced by the feeding regimen, those infants fed breast milk typically stimulate bifidobacteria to become dominant in the first few days of life (1, 53). This dominance of bifidobacteria in the gastrointestinal microbiota of breast-fed infants (90-95% of total microbiota) can continue up through the first two years of an infant's life, and is fundamental for the well-being of the infant (13, 22, 92). As discussed earlier, the explanation for this dominance of bifidobacteria in breast-fed infants is thought to occur due to the natural oligosaccharides, so called prebiotic factors, found in human milk. Promoting this early gastrointestinal colonization with beneficial flora is thus important in infants, especially when breast milk is not available for various reasons. Due to their complexity, oligosaccharides with structures identical to human milk are not yet available as dietary ingredients; however prebiotics similar to human milk oligosaccharides have proved promising. The development of improved infant formulas that emulate the beneficial effects of human milk by supplementing specific prebiotics that selectively stimulate beneficial indigenous bacteria such as bifidobacteria, has gained considerable interest.

Infant and neonate studies are probably the area most extensively researched using GOS as a prebiotic supplement for infant formulas. The majority of the trials, listed in Table 2, have focused on demonstrating the

Table 2. Infant and pregnant mother studies with the prebiotic galactooligosaccharide (GOS)

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
Randomized Control Trials (RCT)	–	Neonates ≤ 37 weeks	4 trials (n=126)	14-33 days	Efficacy and safety of GOS supplementation in reducing the incidence of sepsis and improving physical growth	No significant difference in weight, higher bifidobacteria colony counts, lower pathogenic bacteria colony counts	Srinivasjois <i>et al.</i> (2009)
Double-blind randomized controlled trial (DBRCT)	scGOS/lcFOS (9:1) 6 g/d	Healthy term infants	n=215	first 26 weeks	Explore the effect of infant milk formula (IMF) during the first 26 weeks	No significant differences seen in white blood count, lymphocyte numbers, or immunoglobulins	Raes <i>et al.</i> (2009)
Double-blind randomized	scGOS/lcFOS (9:1) 6 g/d, 6.0E10 <i>Bifidobacterium animalis</i> viable cells	Breast fed and formula fed infants	n=63; 57	4 months	Determine if prebiotics or probiotics should be used to reach a gut flora dominant by bifidobacteria	Prebiotic fed infants tend to have a higher percentage of bifidobacteria counts compared to those of prebiotic fed infants	Bakker-Zierikzee <i>et al.</i> (2005)
DBRCT, Parallel group	scGOS/lcFOS (9:1) 4 g/L plus 3 different prebiotic combinations	Formula fed term infants	n=284	52 weeks	Evaluate infant formulas containing probiotics and synbiotics for safety and tolerance	Incidence of diarrhea significantly lower in prebiotic group, stool frequency significantly higher with synbiotics	Chouraqui <i>et al.</i> (2008)
DBRCT	5 g/L GOS, control	Formula fed term infants	n=159	12 weeks	Determine bifidogenic effects of GOS in a follow-on formula	At weeks 6 and 12 a higher median number of bifidobacteria was observed in the GOS vs. control group	Fanaro <i>et al.</i> (2008)
DBRCT	scGOS/lcFOS (9:1) 8 g/L, control	Hypoallergenic formula fed term infants	n=84	6 months	Analyze the effect of GOS/FOS on the immune response in infants	Significant reduction in plasma level of total IgE, IgG1, IgG2, and IgG3	van Hoffen <i>et al.</i> (2009)
Randomized controlled study	0.24 g/100mL	Formula fed term infants	n=164	3 months	Investigate the effect of infant formula supplemented with low levels of GOS on intestinal micro-flora and fermentation characteristics	Intestinal bifidobacteria, lactobacilli, acetic acid, and stool frequency were significantly increased. stool pH decreased	Ben <i>et al.</i> (2008)
Double-blind controlled	4 g/L GOS, Other prebiotics	Formula fed term infants	n=117	28 days	Investigate effects of prebiotic blends on fecal bacterial populations	No significant changes in bacterial populations for GOS or other blends	Nakamura <i>et al.</i> (2009)

Table 2. Continued

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
DBRCT	scGOS/lcFOS (8:2) increasing dose (max=1.25 g/kg)	Preterm infants	n=113	30 days	Determine the effect of enteral supplementation of prebiotic mixtures on serious infectious morbidity	No significant reduction of serious endogenous infection but trend toward lower incidence	Westerbeek <i>et al.</i> (2010)
<i>Pregnant Mothers:</i>							
DRBCT	GOS/lcFOS (9:1) 3 g; 3 times a day (9 g/d)	Pregnant women vaginal delivery	n=48	week 25-delivery	Determine how supplementation with GOS and lcFOS in the last trimester of pregnancy affects maternal and neonatal gut microbiota	GOS/lcFOS exhibits a bifidogenic effect on maternal gut microbiota but does not transfer to neonates	Shadid <i>et al.</i> (2007)

abilities of GOS to increase fecal bifidobacteria populations. However, a study done by the Nakamura group (2009) did not see any significant differences in change of bacterial populations, but stated the age related differences involved in this study indicated that the GOS blends may have a greater impact on younger infants. With an increase in the fecal bifidobacteria populations, studies performed by Bakker-Zierikzee et al. (2005), Xiao-Ming et al. (2008), and Srinivasjois et al (2009) also exhibited a decrease in fecal pH with more frequent, softer stools. Srinivasjois et al (2009) found that the decrease in fecal pH restricts the growth of potential pathogens, which mimics the pH of breast-fed infants. In addition to the bifidogenic effects, it has been suggested that breast feeding reduces the incidence of allergic disorders in children, in particular atopic dermatitis and wheezing (25). When the effect of GOS/FOS on the immune response in infants was analyzed by E. van Hoffen and colleagues (2009), a significant reduction in the total Ig response was observed. In addition, the GOS/FOS ratio (9:1) modulated the immune response toward cow's milk protein (CMP), leaving the response to vaccination intact. Raes et al (2009) further explored the effects that GOS has on the basal immune parameters during the first 26 weeks of life and found that there was no change when compared to the developing immune system in healthy breast-fed infants. Several of the studies also observed that GOS did not affect weight gain, crying, regurgitation, and vomiting (Table 2). A study done by Shadid and colleagues (2007) also explored how supplementation with GOS in the last trimester of pregnancy affected

maternal and neonatal gastrointestinal microbiota. They found that the supplementation had a bifidogenic effect on the maternal gastrointestinal microbiota that is not directly transferred to the neonates. Results suggested however, that maternal microbiota plays a role in the initial colonization of the infant gastrointestinal tract during the first days of life, as assessed by the high similarity index (SI; 60%). The continued research on infants shows that the incorporation of manufactured prebiotics such as GOS can be a useful addition to formula feeds in order to replicate some of the functional attributes associated with breast milk, particularly its bifidogenic effects; especially with breast milk is not available.

Healthy adults. Since gastrointestinal health can be controlled artificially (introduction of prebiotics), the diet might be the most important regulating factor. As infants, the number of bifidobacteria is the dominant bacterial group, even for bottle-fed infants now that supplementation of prebiotics has been explored. After solid food introduction and weaning from formula or breast milk, the number of bifidobacteria decrease dramatically as bacteroides and firmicute groups become dominant with advanced aging. Due to the beneficial effects that bifidobacteria exert on their host, as discussed earlier, efforts to increase bifidobacteria populations in adult humans are rapidly growing. Introduction of functional foods, in particular prebiotics, has taken the attention of diet supplementation to a more advanced level beyond human milk oligosaccharides. Studies are no longer just focusing on effects in infants, but rather are turning

their attention to the bifidogenic effects seen in adults, and the possible correlation that exists between increased bifidobacteria and health.

Several studies involving healthy human adults have been performed to determine the effects that GOS has on the gastrointestinal microbiota (Table 3). Studies performed by Ito et al (1990; 1993), Alles et al (1999), Depeint et al (2008), Davis et al (2010), and Alander et al (2001) showed that GOS greatly increased bifidobacteria populations and in some cases exhibited a linear-like relationship between the amount of GOS and the number of bifidobacteria in the feces. Effects of GOS on blood lipid concentrations, glucose absorption, and calcium and nonheme-iron absorption were also studied in humans. W van Dokkum and colleagues (1999) showed that 15g of GOS was well tolerated with subjects however, beyond increased flatulence the GOS effects were limited. They observed not cholesterol lowering effects and there was no alteration in blood lipid concentration or glucose absorption. Similar results were observed by van den Heuvel and colleagues (1998), as they did not observe any affect on iron or calcium absorption with the consumption of 15g/day of GOS. An additional study by van den Heuvel et al (2000) explored the effects of GOS on calcium absorption in postmenopausal women and observed an increase in calcium absorption by 16% however, this was not accompanied by increased urinary calcium excretion. This observation implies that GOS may indirectly increase calcium uptake by the bones and/or inhibit bone resorption in postmenopausal women. Research done by Davis et al (2010) showed that a bifidogenic dose

Table 3. Healthy adult studies with the prebiotic galactooligosaccharide (GOS)

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
Latin square randomized double-blind diet controlled	3 treatments of inulin, FOS, and GOS (15 g/d)	Healthy Men	n=12	12 weeks	Study effect of 15 g/d nondigestible oligosaccharides	For GOS; significantly higher fecal wet weight, acetic acid. Significantly lower fecal dry weight. Increase in flatulence	van Dokkum <i>et al.</i> (1999)
Double-blind randomized cross-over study	20 g/d TOS	Postmenopausal women	n=12	37 days	Investigate whether TOS stimulates true Ca absorption in postmenopausal women with decreased efficiency of Ca absorption	Increased Ca absorption observed due solely to TOS	van den Heuvel <i>et al.</i> (2000)
Randomized cross-over study	3 treatments of inulin, FOS, and GOS (15 g/d)	Nonanemic men	n=12	12 weeks	Effects of nondigestible oligosaccharides on metabolism/absorption of calcium or iron in humans	No significant differences were observed with respect to iron or Ca absorption in any of the treatments	van den Heuvel <i>et al.</i> (1998)
Single-blind cross-over study	52% GOS 0, 2.5, 5, or 10 g/d	Healthy adults	n=12	7 weeks	Effects of GOS on microflora, stool weight, abdominal tolerance, and laxative similarities	Linear relationship between amount of GOS and increase in bifidobacteria. Lactobacilli slightly increased with dose	Ito <i>et al.</i> (1990)
Single-blind randomized	93% GOS 0, 2.5, 5, or 10 g/d	Healthy adults	n=18	16 weeks	Determine the effect of different doses of GOS on the fecal microbiota of healthy adults with a focus on bifidobacteria	Significant increase of bifidobacteria was seen after 5.0 g/d in 50% of the subjects.	Davis <i>et al.</i> (2010)
Double-blind randomized cross-over study	15 g/d GOS	Japanese men	n=12	6 days	Effects of GOS after ingestion on the human microflora and metabolism	Bifidobacteria and Lactobacilli significantly increased. Bacteroides and Candida significantly decreased. Bifidobacteria percentage of total bacteria increased.	Ito <i>et al.</i> (1993)
Parallel single-blind	7.5 g/d or 15 g/d	Healthy adults	n=18 (W) n=22 (M)	3 weeks	Compare the effect of two doses of GOS on the gut microbiota composition	Increase in bifidobacteria was seen but not significant. No other significant changes	Alles <i>et al.</i> (1999)

Table 3. Continued

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
Randomized parallel-design	GOS/probiotic mix 3.8 g/d GOS to mix	Healthy adults Male wistar rats	n=38 n=40	8 weeks	Investigate the effects of prebiotic supplementation on a probiotic bacteria mix	There was not significant effect with the rats on bacteria population. Increase in bifidobacteria numbers observed in humans but not significant	Tiihonen <i>et al.</i> (2008)
Double-blind cross-over study	2.5 g/d of GOS or FOS	Healthy adults	n=15	15 weeks	Determine the effect of GOS containing biscuits on the composition and activity of the fecal microflora	Increased metabolic activity but not necessarily increased bifidobacteria numbers. Increased staining in <i>Bifidobacterium adolescentis</i>	Tannock <i>et al.</i> (2004)
Randomized Single blinded study	8.1 g/d GOS with <i>B. lactis</i> Bb-12	Healthy adults	n=30	6 weeks	Further develop the PCR-ELISA methods to follow fluctuations in bifidobacteria	Changes detected were not consistent	Malinen <i>et al.</i> (2002)
Randomized Single blinded study	8.1 g/d GOS with <i>B. lactis</i> Bb-12	Healthy adults	n=30	6 weeks	Investigate the effect of GOS and/or the probiotic strain on composition of indigenous bifidobacteria populations using PCR-DGGE	5 adults showed changes in the DGGE profile of bifidobacteria. The prebiotic did not prolong the persistence of the probiotic strain	Satokari <i>et al.</i> (2001)
Randomized Single blinded study	8.1 g/d GOS with <i>B. lactis</i> Bb-12	Healthy adults	n=30	6 weeks	Effect of GOS-containing syrup on colonization and persistence of <i>B. lactis</i> Bb-12 in the gastrointestinal tract	Mean numbers of bifidobacteria increased slightly in all subjects. Probiotic strain was not enhanced for survival or persistence by GOS	Alander <i>et al.</i> (2001)
Double-blind randomized cross-over study	0.0, 3.6, and 7.0 g/d GOS from <i>B. bifidum</i> 7.0 g/d industrial GOS	Healthy adults	n=59	4 weeks	Access prebiotic potential of GOS produced through β -galactosidases from the probiotic <i>B. bifidum</i> against industrial GOS	Probiotic GOS significantly increased bifidobacteria numbers after 7.0 g/d. Significant relation to bifidobacteria proportion and probiotic GOS dose observed	Depeint <i>et al.</i> (2008)

Table 3. Continued

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
DBRCT	10 g/d of GOS powder	Healthy adults	n=8	21 days	Assess tolerance of GOS and the effect that 10 g/d administration has on fecal concentrations of bacteria	Bifidobacteria concentration was significantly higher after day 7, 14, 21 compared to day 1. Breath H ₂ was significantly lower on day 7, 14, 21 compared to day 1	Bouhnik <i>et al.</i> (1997)
DBRCT Parallel study	7 non-digestible carbohydrates (NDCH) Including GOS 2.5, 5.0, 7.5, or 10 g/d	Healthy adults	n=200	5 weeks	Determine the bifidogenic potential of different NDCH used in human diets and determine a dose response	GOS was found to be bifidogenic. A linear dose relationship was found	Bouhnik <i>et al.</i> (2004)
DBRCT	15 g/d GOS	Healthy adults	n=12	2 weeks	Determine fecal frequency and gastrointestinal symptoms associated with ingestion of GOS containing yogurt	Defecation frequency and flatulence increased Fecal bifidobacteria did not show an increase	Teuri <i>et al.</i> (1998)

dependent response occurred in half of the subjects, indicating a possible 'responders' effect in healthy adults. This 'responders' and 'non-responders' theory can be explained by the presence or absence of specific bifidobacteria strains capable of using GOS as a growth substrate, however what actually differentiates these two groups is still unknown. In several of the studies, a prebiotic effect was not observed at GOS dosage levels below 5.0 g (Table 3), which indicates that a level of at least 5.0 g is needed to elicit a bifidogenic effect. All of the studies reported a return to the initial bifidobacteria population once the consumption of GOS was no longer continued, indicating the stability of the gastrointestinal microbiota of adults. This return to the initial levels of microbiota also indicates that continual consumption of GOS, or the selected prebiotic, will be needed in order to maintain the increased levels of bifidobacteria required for health benefits and disease prevention. The GOS, even at levels of 15 g/day, was well tolerated by adults throughout all of the studies, with only a slight increase in flatulence detected. These studies also showed that GOS can be incorporated into powders, liquids, or solid foods and still exhibit the same prebiotic effects, which is beneficial to the food industry as products are continually being developed. The future of prebiotics, especially GOS, is promising for the health of human adults, however further research is needed to fully understand all of the effects and the possible relationships to diseases.

Elderly adults. As life expectancy throughout the world has rapidly risen, heightened attention has been placed on physiologic and health needs of those

persons over the age of 60 years. Aging is associated with changes in the function of many organs and tissues, including the gastrointestinal tract. The gastrointestinal microbiota evolves with age and the composition of the elderly microflora differs from that of younger adults and infants (100). Putrefactive bacteria, such as clostridia and enterobacteria have been reported to increase in several studies, at the expense of more beneficial groups, most importantly bifidobacteria (27, 40, 58, 59). The aging process is also associated with a marked decline in immune function (immunosenescence), which can promote hyporesponsiveness to vaccination and a predisposition to infectious and noninfectious diseases (5). The intake of food and fluid also decreases, as does physical activity in elderly adults. This, in combination with the use of a wide range of medication that elderly people often use, can cause constipation. As described earlier, reports of increased indigenous bifidobacteria in infants and adults have been observed with the supplementation of GOS in the diet. Studies, however, have not fully determined their effect on elderly persons. Recent research has turned its focus to the use of prebiotics, in particular GOS, in order to increase bifidobacteria populations in elderly with hopes of increased immune function as well as relief of constipation.

For elderly human subjects, data is scarce and contradictory for modulation of the gastrointestinal microflora by GOS, however results are promising (Table 4). Studies performed by Ito et al (1993) and Vulevic et al (2008) showed that administration of GOS, at as little as 2.5 g/day, resulted in a

Table 4. Elderly adult studies with the prebiotic galactooligosaccharide (GOS)

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
Single-blind randomized controlled study	2.5 g/d of GOS	Elderly Men	n=12	3 weeks	Determine the effect of GOS on the human fecal microflora on elderly persons who have low indigenous bifidobacteria	Number of bifidobacteria was slightly increased after consumption of GOS	Ito <i>et al.</i> (1993)
Double-blind placebo controlled randomized cross-over study	10 g/d of GOS	Elderly adults self-reported constipation	n=41	56 days	Evaluate the diversity and temporal stability of predominant fecal bacteria populations in elderly suffering from constipation	No effect to diversity and temporal stability of selected bacterial groups was observed. Elderly did exhibit higher numbers of predominant groups than younger adults	Maukonen <i>et al.</i> (2008)
Double-blind placebo controlled randomized cross-over study	5.5 g/d of GOS	Elderly adults	n=44	28 weeks	Assess the effect of a prebiotic GOS mixture on immune function and fecal microflora of elderly adults	GOS significantly increased bifidobacteria, phagocytosis, NK cell activity, and anti-inflammatory cytokine interleukin-10. A significant reduction in pro-inflammatory cytokines was also observed	Vulevic <i>et al.</i> (2008)
Double-blind two period cross-over study	9 g/d of GOS	Elderly adults Females	n=14	6 weeks	Investigate whether a daily intake of 9 g/d of GOS relieves constipation	Weekly defecation frequency was higher and stools were softer during GOS consumption	Teuri <i>et al.</i> (1998)

significant increase in bifidobacteria. Vulevic and colleagues (2008) also observed that at 5.5 g/day, there was a significant decrease in less beneficial bacterial, which suggests that GOS is an attractive option for enhancement of both the gastrointestinal tract and immune system. Another study performed by Maukonen and colleagues (2008), showed contradictory results as consumption of GOS did not significantly affect the diversity or temporal stability of selected bacterial populations. Reasons for this could be associated with the type and purity of the GOS used, or linked to the ability of the selected subjects to respond to the GOS, as discussed earlier. A study by Teuri and colleagues (1998) focused on relieving constipation in elderly through consumption of GOS. They observed a higher frequency of defecation along with softer stool sample. A dose of 9 g/day of GOS relieved constipation by making defecation easier. Due to the fact that laxatives often have unwanted side effects and some humans are able to build up a gastrointestinal tolerance, an alternative for the use of laxatives in elderly to relieve constipation has gained considerable attention. GOS has proven to be a promising alternative as some researchers have suggested that a reduction in the number of bifidobacteria has been related to constipation and thus treatment with GOS increases the number of bifidobacteria can therefore benefit bowel function (36), however further research is still needed.

Gastrointestinal diseases. Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder. Multidisciplinary approaches have been proposed based on what is known of its pathophysiology, however IBS continues

to represent a significant therapeutic challenge (50, 81). Current interest has focused on the role of the gastrointestinal microbiota-mucosa interactions linked to inflammatory and immune processes. With studies finding benefits pointing to the increase in the bifidobacteria populations by the use of the prebiotic GOS, studies are now investigating the efficacy of GOS as therapy in patients with IBS.

The use of GOS as therapy for IBS patients is very recent and not well explored. In an animal study performed by Holma et al (2002), an increase in bifidobacterial numbers was observed however, there was no reduction in inflammatory processes with the consumption of GOS. A clinical trial by Silk et al (2009) found that GOS had bifidobacterial enhancing effects in IBS patients at a dose of 3.5 g/day and 7.0 g/day (Table 5). This increase in the levels of bifidobacteria became similar to those of healthy humans. They also reported that consumption of GOS was effective in alleviating symptoms associated with IBS. These findings, although several more studies are needed, suggest that the prebiotic GOS has the potential to act as a therapeutic agent in IBS, which is a significant step forward.

Table 5. IBS adult studies with the prebiotic galactooligosaccharide (GOS)

Type	Dose	Subjects	No	Length of study	Objectives	Result	Reference
Randomized parallel cross-over study	3.5 or 7.0 g/d of GOS	Rome II positive IBS	n=44	12 weeks	Investigate the efficacy of a novel prebiotic in changing the colonic microflora and improve IBS symptoms	GOS treatment resulted in significantly higher proportions of bifidobacteria. 7.0 g/d resulted in significantly lower C. perfringens and bacteroides populations. Reported improved stool consistency, flatulence, bloating, and composite score symptoms	Silk <i>et al.</i> (2009)

Conclusion

Prebiotics, especially GOS, are commonly being used as functional ingredients in infant formulas as well as a variety of food products. The consumption of GOS has unquestionably been shown to have a wide variety of metabolic consequences in the human gastrointestinal tract. A vast majority of the investigations into GOS and its effects on the human gastrointestinal microflora have shown its selectivity toward only a few bacterial groups, mainly bifidobacteria. This selectivity has usually only been characterized to the genus level, however researchers are beginning to take advantage of the advanced analytical technologies that have become available for detailed community analysis. Utilizing these advanced technologies, the global effect of prebiotic use on microbial community structure will begin to shed light on the possibilities that prebiotic consumption has. Characterizing these populations beyond the genus level will also help in providing which species have distinct health-promoting properties, as it is unlikely that all of the bifidobacteria colonizing the colon do. Future work with well designed human investigations is still needed to establish prebiotic needs. In particular, further studies are needed to establish the potential role of dietary manipulation for allergy/disease prevention, immune modulating effects of prebiotics in babies and in elderly, and to ascertain whether these effects are long lasting. The optimal dose of GOS for specific subject groups also needs to be determined to help in selecting the proper treatment prior to consumption. In addition, processing procedures may alter the prebiotic

carbohydrate profile in food, which could subsequently affect the digestion and fermentation pathways. Therefore, an understanding of whether the prebiotic activity is maintained after food processing treatments is needed to substantiate the use of GOS as a functional food ingredient. GOS as a prebiotic has undoubtedly played a role in the treatment of IBD however, it is important that systematic studies are instigated to test for GOS benefits on all levels, as well as within a variety of age groups. The future holds promise as a considerable amount of prebiotic literature and research is quickly taking off.

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Chapter 2

**A dose dependent impact of prebiotic galactooligosaccharides on the
intestinal microbiota of healthy adults**

**A dose dependent impact of prebiotic galactooligosaccharides on the
intestinal microbiota of healthy adults**

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RUNNING HEAD: galactooligosaccharides and intestinal microbiota

Abstract

The goal of this research was to determine the effect of different doses of galactooligosaccharide (GOS) on the fecal microbiota of healthy adults, with a focus on bifidobacteria. The study was designed as a single-blinded study, with eighteen subjects consuming GOS-containing chocolate chews at four increasing dosage levels; 0, 2.5, 5.0, and 10.0 g. Subjects consumed each dose for 3 weeks, with a two-week baseline period preceding the study and a two-week washout period at the end. Fecal samples were collected weekly and analyzed by cultural and molecular methods. Cultural methods were used for bifidobacteria, *Bacteroides*, enterobacteria, enterococci, lactobacilli, and total anaerobes; culture-independent methods included denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qRT-PCR) using *Bifidobacterium*-specific primers. All three methods revealed an increase in bifidobacteria populations, as the GOS dosage increased to 5 or 10 g. Enumeration of bifidobacteria by qRT-PCR showed a high inter-subject variation in bifidogenic effect and indicated a subset of 9 GOS responders among the eighteen subjects. There were no differences, however, in the initial levels of bifidobacteria between the responding individuals and the non-responding individuals. Collectively, this study showed that a high purity GOS, administered in a confection product at doses of 5 g or higher, was bifidogenic, while a dose of 2.5 gram showed no significant effect. However, the results also showed that

even when GOS was administered for many weeks and at high doses, there were still some individuals for which a bifidogenic response did not occur.

Introduction

The large intestine of humans harbors a complex, cell rich, and diverse microbial community consisting of hundreds of different bacterial species (Eckburg et al., 2005; Moore et al., 1978). Included within this microbiota are organisms whose presence is associated with, or that contribute to the health of the host (Neish, 2009). In particular, bifidobacteria have long been suggested to play an important prophylactic and therapeutic role in colonic health (Leahy et al., 2005). Although these bacteria are present in large numbers in infants, and are the dominant group in breast-fed individuals, they become less numerous after weaning (Fooks and Gibson, 2002; Mackie et al., 1999). Due to the suggested health benefits these bacteria provide to the host, efforts to enrich the bifidobacteria population are now of considerable interest.

One of the primary ways by which the composition of the intestinal microbiota can be modified is via introduction of prebiotics into the diet (Gibson and Roberfroid, 1995; Roberfroid, 1998). Currently, a prebiotic is defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (Roberfroid, 1998). Among the most widely studied and commercially used prebiotics are inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS). The latter refer to a group of oligomeric, non-digestible carbohydrates that are produced from lactose using β -

galactosidases to catalyze transgalactosylation reactions (Macfarlane et al., 2008; Sako et al., 1999). These β -linked glycosides are recalcitrant to digestion by host-secreted enzymes in the small intestine, such that they reach the colon intact. They then become available to those members of the colonic microbiota metabolically equipped to metabolize these specific oligosaccharides (Alander et al., 2001; Ito et al., 1993; Tannock et al., 2004).

Numerous *in vivo* studies have assessed the effect of GOS on the intestinal microbiota of infants (Chouraqui et al., 2008; Fanaro et al., 2008; Nakamura et al., 2009), pre-term infants (Westerbeek et al., 2010), pregnant women and neonates (Shadid et al., 2007), intestinal bowel disease patients (Silk et al., 2009), elderly adults (Maukonen et al., 2008, Sairanen et al., 2007; Teuri and Korpela, 1998), and healthy adults (Alander et al., 2001; Alles et al., 1999; Bouhnik et al., 1997, 2004; Malinen et al., 2002; Satokari et al., 2001; Tannock et al., 2004; Tiihonen et al., 2008; Vulevic et al., 2008). These studies used doses ranging from as little as 2.5 g per day of GOS to as high as 15 g per day, and relied on cultural as well as molecular methods to measure changes in the microbiota. Although significant increases in the bifidobacteria population were observed in several of these studies (Bouhnik et al., 1997, 2004; Depeint et al., 2008; Vulevic et al., 2008), in other studies bifidogenic effects were not apparent (Alles et al., 1999). Differences in the type, purity, and composition of the GOS used in these studies, as well as difference in experimental design and methods

of analysis, have likely contributed to these varying outcomes (Macfarlane et al., 2008).

The goal of this study was to obtain a more detailed understanding of the effect of GOS on the composition of the human gut microbiota and to determine the dose necessary to achieve a prebiotic or bifidogenic effect. Both culture-based and molecular methods were used to enumerate and characterize shift in selected bacterial populations in eighteen blinded subjects that consumed GOS that had been incorporated into a caramel-like, chewable confection product. The experiment was designed such that subjects were given these GOS-containing products in sequentially higher doses (from 2.5 g to 10 g per day) so that the dosage necessary to elicit a bifidogenic effect could be determined.

Materials and methods

Preparation of chocolate chews

Chocolate-flavored chewable candies (chews) containing GOS and control chews (with no GOS) were prepared at the University of Nebraska-Lincoln Food Processing Center. The GOS used was Purimune™, a high purity GOS powder (91.8% on a dry basis) provided by GTC Nutrition (Golden, CO). The balance of the GOS contained lactose (7%), glucose (<1%), and galactose (<0.5%). The chocolate chews were formulated to contain 1.25 g of GOS per 6 g chew.

Additional corn syrup and sucrose were included in the control chews containing no GOS. The formulations of both the GOS and control chew are shown in Table 1. Chews were wrapped individually in wax paper and stored in sealed plastic bags at 20°C. The chews were distributed to subjects on a weekly basis.

Experimental design

The study included 21 healthy human volunteer subjects that were recruited on the University of Nebraska-Lincoln campus. None of the subjects had been on antibiotics or on a vegetarian diet within three months prior to the start of the study or during the study. Subjects were allowed to maintain their normal lifestyles without any additional restrictions on their diets. Two subjects dropped out of the study for reasons unrelated to the experiment and one subject was released from the study due to pregnancy. Thus, a total of eighteen subjects, 13 males and 5 females, between the ages of 19 and 50 years old, completed the study. The study was conducted over a 16 week period. A two-week baseline period (no chews administered) was conducted at the beginning of the study, followed by four sequential testing periods during which chews were administered for three weeks with GOS dosages at levels of 0.0 g, 2.5 g, 5.0 g, and 10.0 g GOS per day. Subjects were blinded in terms of the dose of GOS they received, and instructed to consume eight chews per day during each testing period, with the only difference being the number of GOS-containing chews included in the daily regimen, which could not be differentiated from

control chews. Thus, during the control period, 8 control chews were consumed, and during the 2.5 g treatment period, 2 GOS chews (each containing 1.25 g GOS) and 6 control chews were consumed. The 5 g treatment period included 4 GOS and 4 control chews and the 10 g treatment consisted of 8 GOS chews. A final two-week washout period (no chews) was performed at the end of the fourth testing period. All of the dosages were sequential with no washout periods between dosages. Subjects were asked to report the presence, absence, and severity of gastrointestinal symptoms experienced throughout each week of the study. The symptoms survey was based on previously reported studies (Bouhnik et al., 1997, 2004; Shadid et al., 2007) and included bowel movement, stool consistency, discomfort, flatulence, abdominal pain, and bloating, and were scored on a one (none, normal, good well-being) to five (severe symptoms and discomfort) scale provided as part of weekly subject diaries. The study was approved by the Institutional Review Board of the University of Nebraska.

Collection and processing of fecal samples

Fecal samples were collected weekly from each subject. Each sample was processed within 1 hour of a bowel movement. All fecal samples (1.0 g) were weighed and diluted 10-fold with sterile phosphate buffered saline (PBS; pH 7.0). Samples were homogenized and immediately frozen at -80°C and saved for DNA extraction. Fecal samples (1.0 g) were also immediately introduced into an anaerobic chamber (Bactron IV Anaerobic Chamber, Shel Lab, Cornelius, OR)

and a 10-fold dilution series was made with pre-reduced sterile saline (0.9% NaCl). Aliquots were plated on Brain Heart Infusion Agar (Becton Dickinson; BD, Franklin Lakes, NJ) for total anaerobes (incubated 48 h), Rogosa SL (BD) for *Bifidobacterium* (96 h), and Bacteroides Bile Esculine Agar (BD) for *Bacteroides* (48 h). All plates were incubated anaerobically at 37°C. In addition, the Rogosa SL agar plates that were used to enumerate bifidobacteria were also examined at 48 h to estimate lactobacilli levels. Serial dilutions were also used to plate aliquots aerobically on MacConkey Agar (BD) for enterobacteria (24 h), and Bile Esculin Azide Agar (Acumedia, USA) for enterococci (48 h). Plates were incubated aerobically at 37°C. These organisms were chosen for cultural enumeration based on previous prebiotic and probiotic feeding studies (Tannock et al., 2000, 2004).

The fecal pH was measured in aqueous slurries using an Ag/AgCl pH meter (Accumet Basic AB15pH meter, Fisher Scientific). Statistical analysis was completed using a one-way ANOVA as well as Tukey's post-hoc pair-wise comparison test.

DNA extraction

A 1 mL aliquot of a 1:10 diluted fecal sample in PBS was transferred to sterile bead beating tubes (Biospec products, Bartlesville, OK, USA) containing 300 mg of zirconium beads (0.1 mm). Fecal cells were washed three times in chilled PBS using centrifugation at 6,000 x g for 5 min. Pellets were resuspended in 100

μL of lysis buffer (200 mM NaCl, 100 mM Tris, 20 mM EDTA, 20 mg/mL Lysozyme, pH 8.0) and incubated at 37°C for 30 min. Buffer ASL (1.6 mL) from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added to each sample after the samples were homogenized in a MiniBeadbeater-8 (BioSpec Products, OK, USA) for two min at maximum speed. The DNA was purified from the supernatants using the QIAamp DNA Stool Mini Kit, following the Qiagen kit manufacturer's instructions.

Quantitative real time-PCR

Quantitative real time PCR (qRT-PCR) was performed as described by Martínez et al. (2009) using a Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany) with *Bifidobacterium*-specific primers F: 5'TCGCGTC(C/T)GGTGTGAAAG'3 and R: 5'CCACATCCAGC(A/G)TCCAC'3 (Martínez et al. 2009, Rinttila et al. 2004), with an amplicon size of 243 bp. Standard curves for absolute quantification of bifidobacteria in the fecal samples were prepared using overnight cultures (14 h) of *Bifidobacterium animalis* ATCC 25527^T and *Bifidobacterium infantis* ATCC 15697^T. For each qRT-PCR experiment, a standard curve was prepared, in duplicate, using DNA extracted from cultures at concentrations ranging from 10⁵-10⁸ CFU/mL. Correlation coefficients for all standard curves were above 0.95.

Analysis by PCR-DGGE

PCR-DGGE was performed as described by Martínez et al. (2009). Briefly, the V3 region of the 16S rRNA gene was amplified by PCR using universal primers PRBA338fGC

(5'CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG'3) and PRUN518r (5'ATTACCGCGGCTGCTGG'3) (Ovreas et al., 1997). Denaturing Gradient Gel Electrophoresis (DGGE) was performed as described previously (Walter et al., 2000), using a DCode universal mutation detection system (Bio-Rad, Hercules, USA). Band fragments of interest were excised, repeatedly purified (Walter et al. 2001), and then cloned using the TOPO® TA Cloning® Kit for Sequencing (pCR® 4 TOPO® Vector) (Invitrogen). The QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used to isolate plasmids from transformants, and inserts were sequenced by a commercial provider. Closest relatives of the partial 16S rRNA sequences were determined using the SeqMatch web tool provided through the Ribosomal Database Project (<http://rdp.cme.msu.edu>).

BioNumerics software Version 5.0 (Applied Maths) was used to analyze DGGE profiles. DGGE bands were automatically assigned and densitometric curves were obtained based on the staining intensity profiles generated by the BioNumerics software. Band staining intensities were calculated as a percent of each peak area of the entire fingerprint generated for the individual sample. We have previously determined the reliability of this quantification method by

comparing taxa abundance inferred by DGGE band intensities with those obtained with pyrosequencing of 16S rRNA tags in studies on the hamster microbiota, and received correlations of $r > 0.8$ (Martínez et al., 2009).

Statistical analysis

One-way ANOVA tests with repeated measures were used to determine significance between the different doses of GOS (0, 2.5 g, 5 g, and 10 g) and the control. Baseline/washout samples were combined for the analysis and referred to as “none”. Statistical analysis was performed for the combined data from the eighteen subjects and to identify statistically significant increases of individual subjects. Tukey’s test was used for post hoc pair-wise comparisons.

Results

Digestive tolerance of GOS

All eighteen subjects completed a weekly symptoms diary throughout the duration of the study. These symptoms diaries allowed subjects to rate bowel movement, stool consistency, discomfort, flatulence, abdominal pain, and bloating on a scale of one (none, normal, good well-being) to five (severe symptoms and discomfort). Based on a one-way ANOVA of the data, no significant differences were detected for any of the symptoms between the 0.0 g

GOS control dose and any of the GOS treatments (Table 2). A significant symptom change was observed for flatulence ($p < 0.05$), but only between the baseline and washout and the treatment periods. However, the increase in this score occurred not only for the GOS treatments, but even during consumption of the 0.0 g GOS control period.

Fecal bacteria counts

Cultural enumerations were performed for total anaerobic bacteria and for lactose-fermenting enterobacteria, enterococci, *Bifidobacterium*, and *Bacteroides*. Lactobacilli counts were very low ($< 10^6$ /g) throughout the entire duration of the study, even during treatment periods (data not shown). When the data for each individual subject was analyzed, the results revealed that for some subjects, statistically significant differences in several of these groups were observed following consumption of GOS (data not shown). When the results of all eighteen subjects were pooled together, no significant changes were detected for levels of *Bacteroides*, enterococci, or lactose fermenting enterobacteria. However, ANOVA revealed that GOS induced a modest, but statistically significant increase of bifidobacteria compared to the control treatment (Table 3). This bifidogenic effect occurred when subjects had consumed the 5 g dose of GOS, and a further increase in dose to 10 g of GOS was not significant when compared to the 5 g dose. In contrast, however, the 10 g dose did result in a significant increase in total anaerobes compared to the 2.5 g dose. In addition,

we observed that the bacterial populations for all groups were similar during the baseline and washout periods.

The pHs of all of the fecal samples (288) were determined. All but two of the samples had pH values between 6.0 and 8.0, and there were no significant treatment differences in pH observed over the period of the study (data not shown).

Genus specific qRT-PCR for enumeration of bifidobacteria

The culture analysis indicated that a bifidogenic effect occurred due to consumption of GOS, and that this effect was detectable at doses of 5 g and 10 g, with no significant differences between the two high doses. In order to confirm these findings without a potential cultivation bias, cell numbers of bifidobacteria in fecal samples were quantified by genus-specific qRT-PCR. As shown in Figure 1a, the *Bifidobacterium* population in the eighteen subjects increased with the inclusion of chews containing different amounts of GOS. As before for cultural enumeration, this increase reached statistical significance when 5 g and 10 g of GOS were consumed ($p < 0.001$). The analysis also showed major differences in the dose response relationships in individual subjects. In total, *Bifidobacterium* numbers were significantly increased by GOS consumption in nine of the eighteen subjects as analyzed by ANOVA. Figure 1b shows the numbers of bifidobacteria in these nine “responders”. This data showed an equivalent

gradual increase of bifidobacteria with dose, with no significant differences between 5 and 10 g of GOS.

To determine if the baseline *Bifidobacterium* population influenced the prebiotic effect, we compared the initial bifidobacteria levels between responders and non-responders. The Student's *t* test did not reveal any significant differences between these groups (data not shown), indicating that initial number of bifidobacteria did not determine whether any specific individual was a responder or non-responder. In contrast, the baseline cell count of bifidobacteria in subjects was a major determinant for the bifidogenic effect when this effect was based on the difference in actual numbers from the baseline to the average of the 5 and 10 g treatments. As shown in Figure 2A, initial levels of bifidobacteria directly correlated with the increase of bifidobacteria numbers. However, the bifidogenic effect, expressed as the "log increase", was inversely correlated with the initial bifidobacteria levels (Figure 2B). In other words, subjects with low numbers of bifidobacteria had a higher potential for the prebiotic to induce a 100-1000 fold increase, while subjects that already possessed high levels of bifidobacteria were able to achieve an even higher increase in absolute numbers.

Characterization of total fecal bacterial populations by PCR-DGGE

To obtain a broader assessment of the impact of GOS on the fecal microbiota, we used a universal PCR-DGGE approach to determine the dynamics of the community fingerprints. These analyses revealed a high level of stability among

the gut microbiota in all of the subjects. The DGGE gels corresponding to the eight subjects with the most pronounced changes in staining intensities upon consumption of GOS are shown in Figure 3. Quantification of DGGE band intensities was then performed using BioNumerics software, as previously reported (Martínez et al., 2009), revealing several major effects (Table 4).

The most consistent alteration in band staining intensity resulting from consumption of GOS was a band at the bottom of the DGGE gels (labeled as C, G, H, I, and L), that was present in five subjects, 2, 4, 14, 15, and 17 (Figures 3A and 3B). Excision of the band and subsequent purification and DNA sequencing revealed that the band corresponded to *Bifidobacterium adolescentis* (Table 5). The staining intensity of this band clearly showed a dose dependent increase (Table 4), although differences were observed between subjects with respect to the effective dose (ranging from 2.5 – 10 g). However, when the band intensity values from these five subjects were averaged, the results revealed that a bifidogenic effect occurred only when the GOS dose reached 10 g (Figure 3C).

Collectively, the abundance of *B.adolescentis*, as determined by staining intensity, was remarkably quantitative and highly correlated to cell numbers as determined by qRT-PCR (Figure 3D). Also, as shown in Figure 3 and Table 4, the increase in *B. adolescentis* was reversible and returned to the baseline level within a week of wash out. Consumption of GOS also resulted in several other reversible alterations in the fecal microbiota; however, most of these alterations

related to a decrease in staining intensity of bands that corresponded to different colonic microorganisms (Table 5).

Discussion

The ability of GOS to effect changes in the microbiota of the human intestinal tract was first reported in 1993 (Ito et al., 1993). Although increases in the bifidobacteria population in test subjects were reported in this and many other studies (Bouhnik et al., 1997, 2004; Depeint et al., 2008; Vulevic et al., 2008), in other investigations a bifidogenic effect of GOS was not detected (Alles et al., 1999; Malinen et al., 2002). Variations in the GOS type, dosage, the delivery vehicle, and in the experimental design likely account for some of these different outcomes. However, the methods of analysis may have also contributed to these differences, especially when enumeration was based primarily on cultural methods.

In this study, three independent techniques (group-specific culturing, qRT-PCR, and PCR-DGGE) were used to study the impact of different doses of a highly pure source of GOS on the human gut microbiota in eighteen healthy subjects. The GOS was incorporated into chewable confections, delivered in sequentially higher doses, and all of the subjects were blinded. Samples were obtained and analyzed three times during each of the four treatment periods, and twice during

both the baseline and washout periods. In general, the cultural enumeration results during the baseline period were consistent with other reports utilizing similar techniques to quantify members of the gastrointestinal microbiota (Tannock et al., 2000, 2004). The low levels of lactobacilli in most of the subjects throughout the study were also consistent with previous observations (Eckburg et al., 2005; Tannock et al., 2000; Walter, 2008). Importantly, however, all three methods clearly confirmed a highly significant bifidogenic effect of GOS that was quickly reversed when GOS consumption had ended. In addition, the results indicated that the effect was dose-dependent. Although we detected a modest increase of bifidobacteria numbers by cultural and qRT-PCR methods at a daily dose of 2.5 g of GOS, it required 5 g per day to achieve statistically significant higher numbers of bifidobacteria, relative to the control. A further increase in the dose from 5 to 10 g of GOS did not lead to an additional increase in the absolute number of bifidobacteria when determined by both culture and qRT-PCR (Table 3, Figure 1).

Collectively, the data obtained in this study suggests that the dose does influence the bifidogenic effect of a prebiotic food product. However, it has previously been suggested that the daily dose of a prebiotic is not a determinant of the prebiotic effect (Gibson et al., 2004; Roberfroid, 2007). According to this argument, the prebiotic effect is influenced by the starting number of bifidobacteria in the subjects prior to administration of the prebiotic, such that the

larger the number of initial fecal bifidobacteria present in an individual, the greater is the potential for a bifidogenic effect. Thus, increases in bifidobacteria, in absolute numbers, in response to prebiotics directly correlate with the initial number, but inversely correlate with log transformed increases. Our results support this conclusion, as we observed that the greatest bifidogenic response to GOS, in absolute numbers, occurred in subjects having the highest initial bifidobacteria levels, while a higher log increase was observed in subjects with low initial numbers (Figure 2). However, our data still clearly indicates that the dose of GOS was an important determinant of the prebiotic effect. As shown above, when all eighteen subjects were considered, a significant bifidogenic effect required 5 g of GOS, relative to the control, whereas 2.5 g was not sufficient. The data suggests that a minimum or 'threshold' dose may exist below which a prebiotic effect is not observed. Accordingly, in a previous study, 2.5 g of GOS did not lead to an increase in cell numbers of bifidobacteria (Tannock et al., 2004). However, our data does also indicate that there may also be a dose at which no additional bifidogenic effect is observed (i.e., above 5 g in our study). Above this threshold, the dose of a prebiotic is indeed not a determinant of the prebiotic effect, as suggested by Gibson and Roberfroid (Gibson et al., 2004; Roberfroid, 2007). We suggest, however, that the determination of a prebiotic dose or threshold, as determined in this study, is valuable for dietary recommendations, as this amount constitutes a minimum dose by which a significant bifidogenic effect can be achieved in a population of subjects.

Although all three methods of analysis showed that GOS could elicit a bifidogenic response when the results from the 18 subjects were pooled, we also observed considerable individual variations. Thus, only 50% of the subjects showed a statistically significant increase of bifidobacteria following GOS consumption. What differentiates these responders from non-responders is unknown, but we suggest that one explanation may simply be due to the presence or absence of specific *Bifidobacterium* strains capable of using that prebiotic as a growth substrate. Thus, responders may harbor a greater proportion of GOS-fermenting bifidobacteria among the gut microbiota, compared to non-responding individuals. Although we did not detect differences in the initial numbers of bifidobacteria in responders and non-responders, individuals in the latter group might nonetheless lack specific GOS-utilizing strains. In addition, although the effective bifidogenic dose was 5 g for the subjects on average, subject-specific dose-response relationships were also detected (data not shown).

In this study, we incorporated the GOS into a chewable confection product to simulate a relevant means of delivery. Preliminary evaluations indicated that the control and GOS-containing chews were indistinguishable based on appearance, flavor, and chewiness. All of the GOS dosage levels used in this study, including the 10 g per day dose, were well tolerated, based on the absence of any adverse effects compared to the control chews as reported in the symptoms diaries. Although subjects did report an increase in the flatulence score, significance

occurred between the baseline period and even the control (no GOS) treatment, suggesting that this outcome was due either to a placebo effect or was caused by another component of the chew. Moreover, no differences in flatulence were reported between any of the treatment doses. The general tolerance of GOS and the absence of undesirable side-effects at these dosages has previously been reported (Ito et al., 1990).

Several reports have shown that DGGE is an effective method to assess the effect of GOS consumption on the stability and diversity of the human intestinal microbiota (Maukonen et al., 2008; Tannock et al., 2004). In the latter study, the DNA-DGGE profiles were not altered in healthy adults following consumption of GOS-containing biscuits (2.5 g per day for three weeks), although changes in the RNA-derived DGGE profiles were observed. The RNA-DGGE fragments whose intensity had increased during GOS were sequenced and subsequently assigned to *Bifidobacterium adolescentis* and/or *Colinsella aerofaciens*. In the current study, DGGE analysis provided evidence that consumption of GOS induced compositional alterations in the fecal microbiota of a majority of subjects. Moreover, the DGGE results showed that the changes in the microbiota were selective, with the most consistent alteration detected being an increase in the intensity of a band corresponding to *Bifidobacterium adolescentis*.

Although specific health benefits have not yet been causally linked to particular bacterial populations in the human gut, bifidobacteria are generally considered to

be health-promoting organisms and constitute one of the main groups of organisms targeted by prebiotics. In this study, we provide evidence that a minimum dose of 5 g of GOS per day induced significant alterations in the gut microbiota in healthy human adults, mainly by increasing the number of bifidobacteria. We argue that dose-response studies such as the one presented here might enable better dietary recommendations on an effective dosage of prebiotics, especially when incorporated directly into foods. However, as shown in this study, it appears that even when GOS is administered for many weeks and at high doses, there may still be some individuals for which a bifidogenic response does not occur.

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Table 1. Composition (%) of chocolate chews

Ingredient	Control Chocolate Chew	GOS Chocolate Chew
Water	11.62	11.54
Sugar	27.35	19.42
GOS (Purimune)	0.00	23.40
Corn Syrup	44.84	31.83
Palm Kernel Oil	7.62	5.41
Chocolate Liquor (1/2 Bakers)	7.58	7.44
Lecithin	0.55	0.53
Vanilla	0.44	0.43

Table 2. Mean \pm standard deviations of weekly symptoms. Reported on a scale of 1 (best) to 5 (worst).

	Baseline	0.0 g	2.5 g	5.0 g	10.0 g	Washout
Bowel Movement	1.42 \pm 0.55	1.57 \pm 0.61	1.44 \pm 0.55	1.39 \pm 0.51	1.46 \pm 0.61	1.42 \pm 0.79
Stool consistency	1.56 \pm 0.64	1.63 \pm 0.68	1.54 \pm 0.68	1.54 \pm 0.73	1.57 \pm 0.65	1.50 \pm 0.84
Discomfort	1.42 \pm 0.69	1.48 \pm 0.60	1.56 \pm 0.57	1.44 \pm 0.65	1.52 \pm 0.73	1.14 \pm 0.38
Flatulence	1.52 \pm 0.78	1.83 \pm 0.75*	1.85 \pm 0.79*	1.86 \pm 0.75*	2.07 \pm 0.88*	1.25 \pm 0.55
Abdominal pain	1.17 \pm 0.38	1.31 \pm 0.49	1.33 \pm 0.40	1.30 \pm 0.50	1.30 \pm 0.60	1.14 \pm 0.41
Bloating	1.14 \pm 0.33	1.39 \pm 0.75	1.43 \pm 0.47	1.30 \pm 0.65	1.48 \pm 0.90	1.08 \pm 0.26

*Significant differences detected by ANOVA ($p < 0.05$) between the GOS and baseline and washout treatments.

Tukey's post-hoc test did not detect significant differences in pair wise comparisons.

Table 3. Enumeration of bacterial groups through culturing

Bacterial group	Log 10 cfu/g feces (Mean \pm SD)					
	Baseline	0.0 g	2.5 g	5.0 g	10.0 g	Washout
Lactose fermenting	5.60 \pm 1.14	5.68 \pm 1.07	5.64 \pm 0.86	5.18 \pm 1.26	5.59 \pm 0.85	5.78 \pm 1.17
Enterobacteria						
Enterococci	5.02 \pm 0.99	5.02 \pm 1.07	4.95 \pm 0.99	4.67 \pm 0.93	4.70 \pm 0.90	5.13 \pm 1.10
Bifidobacteria	9.32 \pm 0.79	9.48 \pm 0.73	9.60 \pm 0.80	9.76 \pm 0.48*	9.83 \pm 0.56***	9.42 \pm 0.52
Bacteroides	9.56 \pm 0.37	9.58 \pm 0.37	9.59 \pm 0.35	9.47 \pm 0.32	9.53 \pm 0.35	9.53 \pm 0.33
Total anaerobes	10.19 \pm 0.28	10.19 \pm 0.20	10.11 \pm 0.23	10.24 \pm 0.15	10.35 \pm 0.16**§§§	10.19 \pm 0.21

Significantly different to 0.0 g: * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

Significantly different to 2.5 g: §§§ (p < 0.001)

Table 4. Ratio of staining intensities of major bands as proportion of total fingerprint intensity (%) and results of sequence analysis of selected bands.

		Mean band intensity (± SD)					
		Increasing significance					
Subject	DGGE fragment	Baseline	0.0 g	2.5 g	5.0 g	10.0 g	Washout
2	C	3.68 ± 0.02	2.76 ± 0.009	4.87 ± 0.01	6.67 ± 0.03	11.89 ± 0.04***§	2.93 ± 0.02
4	F	3.13 ± 0.02	1.23 ± 0.001	3.83 ± 0.02	3.33 ± 0.005	6.96 ± 0.02**	1.39 ± 0.002
	G	1.46 ± 0.007	3.94 ± 0.02	2.70 ± 0.004	5.51 ± 0.01	9.75 ± 0.03***§§	1.21 ± 0.003
14	H	3.41 ± 0.16	6.40 ± 0.06	8.57 ± 0.02	7.41 ± 0.008	8.06 ± 0.02	0.69 ± 0.01
15	I	0.00 ± 0.00	0.00 ± 0.00	1.48 ± 0.002*	2.53 ± 0.01**	2.55 ± 0.002**	0.00 ± 0.00
17	L	0.92±0.002	1.73±0.01	1.48±0.01	5.15±0.007	10.00±0.03***§§§†	0.60±0.002
Decreasing significance							
1	A	11.91 ± 0.03	10.73 ± 0.03	2.79 ± 0.05*	0.12 ± 0.001**	0.19 ± 0.002**	1.15 ± 0.01
2	B	5.07 ± 0.01	3.50 ± 0.01	3.17 ± 0.01	2.88 ± 0.003	1.03 ± 0.002	2.51 ± 0.01
3	D	6.51 ± 0.02	7.06 ± 0.03	3.42 ± 0.04	1.30 ± 0.02	0.001 ± 0.002	4.50 ± 0.004
4	E	1.88 ± 0.001	1.66 ± 0.005	3.35 ± 0.003	2.69 ± 0.02	0.35 ± 0.0009§	2.63 ± 0.02
16	J	9.74 ± 0.03	8.00 ± 0.03	7.15 ± 0.009	4.13 ± 06	4.10 ± 0.005	4.33 ± 0.004
17	K	8.70 ± 0.002	3.68 ± 0.04	6.79 ± 0.02	2.80 ± 0.02	0.63 ± 0.005	2.83 ± 0.004

Significantly different to 0.0 g: * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

Significantly different to 2.5 g: § (p < 0.05), §§ (p < 0.01), §§§ (p < 0.001)

Significantly different to 5.0 g: † (p < 0.05)

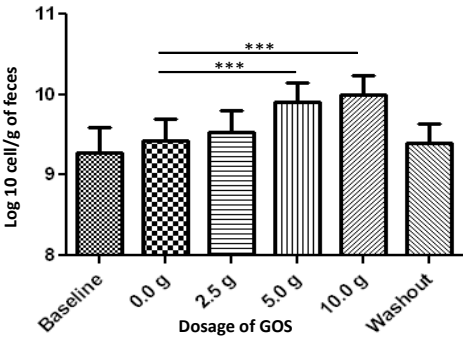
Subjects 3, 14, 2B, 16, and 17K are included because they are approaching significance (p < 0.05) at 10 g compared to 0.0 g.

Table 5. Identification of band fragments in DGGE gels

Subject	Band fragment	Closest related Genbank sequence (% similarity between DGGE fragment and Genbank sequence)
1	A	<i>Ruminococcus</i> uncultured bacterium; 29A-b4; DQ905715 (99%)
2	B	<i>Lachnospiraceae</i> uncultured bacterium; RL197_aah88b02; DQ794455 (100%)
	C	<i>Bifidobacterium adolescentis</i> ; E-981074T; nru-5; AF275882 (100%)
3	D	<i>Bacteroides</i> uncultured bacterium; NO48; AY916250 (100%)
4	E	<i>Bacteroides uniformis</i> (T); JCM 5828T; AB050110 (100%)
	F	<i>Bacteroides dorei</i> (T); JCM 13471; 175; AB242142 (100%)
	G	<i>Bifidobacterium adolescentis</i> ; E-981074T; nru-5; AF275882 (99%)
14	H	<i>Bifidobacterium adolescentis</i> ; E-981074T; nru-5; AF275882 (100%)
15	I	<i>Bifidobacterium adolescentis</i> ; E-981074T; nru-5; AF275882 (100%)
16	J	<i>Ruminococcaceae</i> uncultured bacterium; RL185_aan85a07; DQ825073 (100%)
17	K	<i>Ruminococcus</i> uncultured bacterium; B086; DQ325583 (97%)
	L	<i>Bifidobacterium adolescentis</i> ; E-981074T; nru-5; AF275882 (100%)

Figure 1. Bifidogenic effect of GOS as determined by qRT-PCR for all eighteen subjects (**A**) and for the 9 responders (**B**). Significance (by ANOVA) is indicated at either $p < 0.05$ (*) or $p < 0.001$ (***)).

A



B

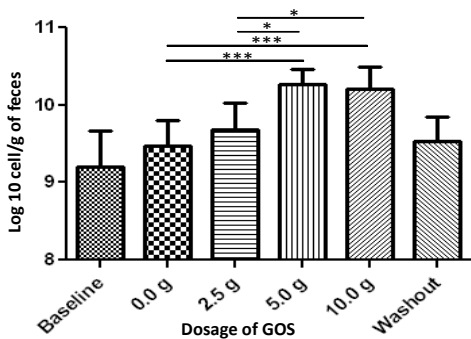
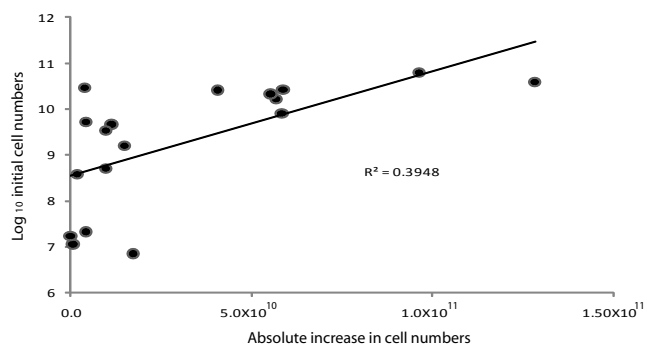


Figure 2. Correlation of initial bifidobacteria levels (baseline) and the increase of bifidobacteria by GOS feeding (from the baseline to the average of the 5 and 10 g dose levels) as measured by absolute numbers (**A**) and by log increase (**B**).

A



B

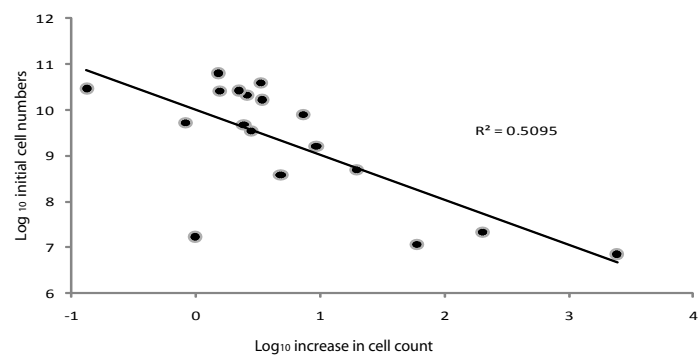
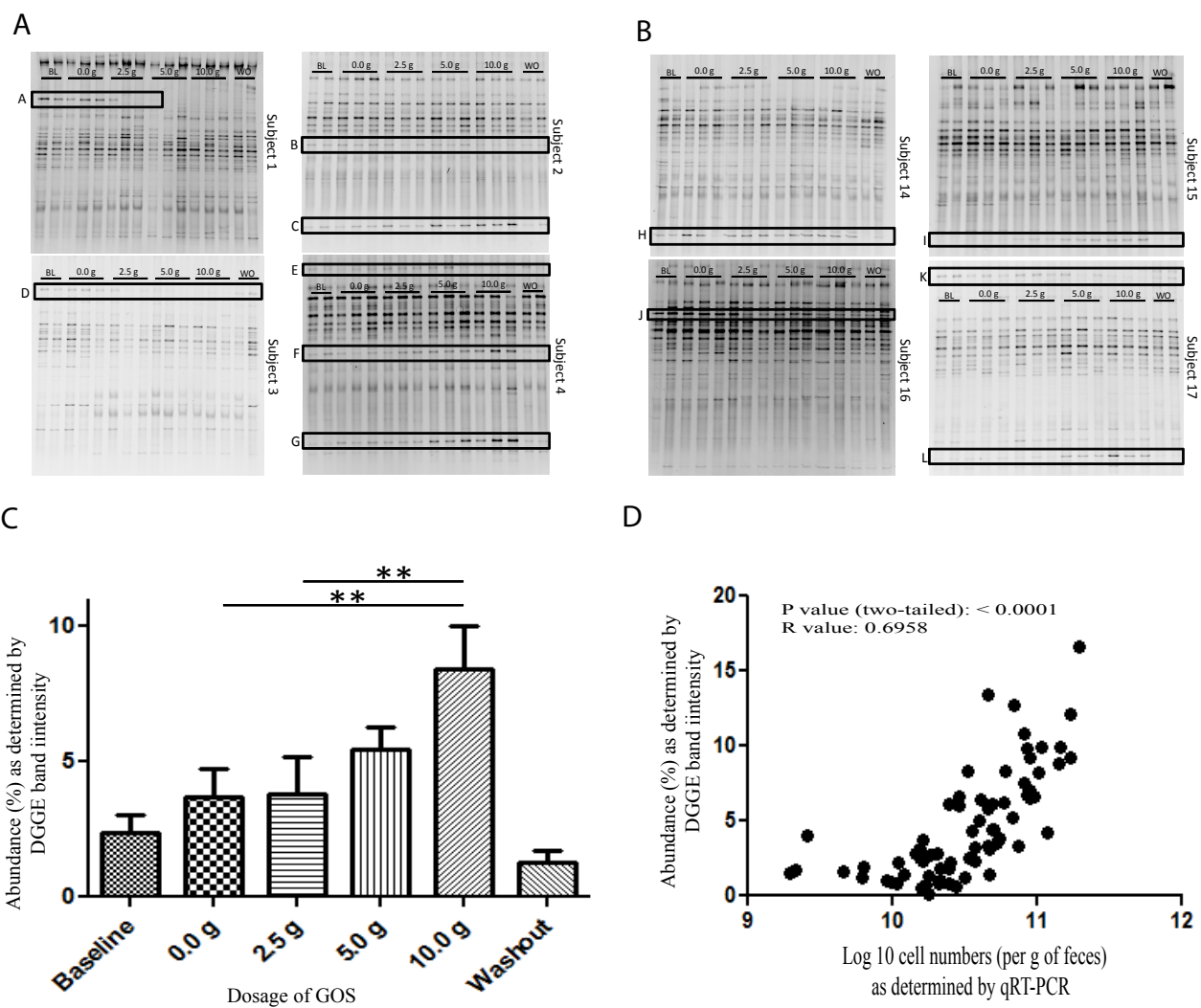


Figure 3. DGGE analysis of fecal microbiota of subjects 1, 2, 3, and 4 (**A**) and 14, 15, 16, and 17 (**B**) by DGGE. Bands that were significantly affected by the GOS treatments are outlined. Abundance scores, as measured by DGGE band intensities from bands, C, G, H, I, and L, as a function of GOS doses (**C**). Correlation of *Bifidobacterium adolescentis* band intensities from subjects 2, 4, 14, 15, and 17 for all time points to cell numbers, as measured by *Bifidobacterium* genus-specific qRT-PCR (**D**).



Chapter 3

**Community sequencing reveals that galactooligosaccharides consumption
results in a highly specific bifidogenic response**

**Community sequencing reveals that galactooligosaccharides consumption
results in a highly specific bifidogenic response**

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RUNNING HEAD: galactooligosaccharides and intestinal microbiota

ABSTRACT

The goal of this research was to gain a community wide perspective of the impact of GOS on the fecal microbiota of healthy human subjects using high throughput multiplex community sequencing of 16S rRNA tags. Eighteen healthy human subjects consumed GOS containing caramel-like confectionary products for twelve weeks, with four increasing dosages of GOS. Multiplex sequencing of the 16s rRNA tags revealed that GOS induced significant compositional alteration in the fecal microbial populations by increasing the phyla Actinobacteria. At the species level, the changes evoked by GOS resulted in increases of six of the *Bifidobacterium* species, including *B. adolescentis*, *B. longum*, and *B. catenulatum*. The population shifts caused by consumption of 10 g of GOS were numerically substantial, leading for example, to a ten-fold increase in bifidobacteria in four subjects, enriching them to 18-33% of the fecal microbial community, and a five-fold increase in seven additional subjects. Moreover, this increase in bifidobacteria abundance, to greater than 20% in some individuals, was generally at the expense of only one group of bacteria, namely the genus *Bacteroides*. The responses to GOS and their magnitudes varied between individuals, and they were reversible and tightly associated with the increasing dosage of GOS. Our results demonstrate that GOS is remarkable for its ability to enrich specifically for bifidobacteria in human fecal samples, although it is utilized by a wide variety of bacterial inhabitants of the intestinal tract when studied in vitro.

INTRODUCTION

It has become increasingly recognized that the gastrointestinal microbiota plays a critical role in human health (13). The composition and activity of this microbiota affect nutrient utilization and adsorption, the development and maturation of the immune system, and resistance to infections (24, 43, 47). Aberrations in the gut microbiota have been linked several complex diseases, including inflammatory bowel disease (13, 19, 34), colitis (27, 33), osteoporosis (1,35), obesity, atherosclerosis and cardiovascular disease (32, 44, 14), type 2 diabetes (9), colorectal cancer (21, 55,20), arthritis, and allergic diseases (26, 30, 31, 42, 59). Moreover, the discovery that it is possible to effect changes in the intestinal microbiota by relatively small dietary modifications (35) has led to the suggestion that these aberrations or imbalances can be corrected and host health improved (22).

One strategy by which to modulate composition and metabolism of the intestinal microbiota are prebiotics. Prebiotics are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (45). Several of these carbohydrates are used commercially in foods, including inulin, fructooligosaccharides (FOS), resistant starch, and galactooligosaccharides (GOS). There is now convincing *in vivo* evidence showing that prebiotics can promote growth of bifidobacteria in the intestinal tract of infants and adults (56). For GOS in particular, 2 to 3 log increases in the

number of bifidobacteria have been reported (15). However, the human gut microbiota is composed of hundreds of species (22), and the impact of prebiotics on other members of the intestinal microbiota and the community structure in general is less well understood.

. The specificity of prebiotic substrates was initially attributed to their selective fermentation in the intestinal tract. Several surveys have revealed that several species of *Bifidobacterium*, as well as *Lactobacillus*, are able to ferment prebiotic substrates. Interestingly, however, in monoculture, several colonic bacteria other than bifidobacteria have been reported to utilize prebiotics as an energy or carbon source, including species of *Clostridium*, *Enterococcus*, *Bacteroides*, and *Escherichia*. These groups of bacteria have previously not been reported to be enriched through prebiotics in human trials, and other mechanisms have been proposed for the relatively specific bifidogenic effect of prebiotics, such as tolerance to SCFA and acidification and the ability to adhere to prebiotic substrates (12). However, most studies on the in vivo specificity of prebiotics applied methodologies with limitations in their ability to address the question on how specific prebiotics are. These studies relied on either cultural enumeration methods that fail to detect the majority of microbial species present in the human gut (2, 8, 16, 29), or they used molecular methods that are restricted by focusing on selected bacterial groups (qRT-PCR, fluorescent in situ hybridization (FISH;16) or suffer from a small dynamic range (DGGE; 52,15, T-RFLP). Several of these studies showed that the prebiotic response was not

completely restricted to bifidobacteria. For example, Tannock and co-workers showed that FOS increased staining intensities of bands corresponding to *Bifidobacterium adolescentis* and *Colinsella aerofaciens* (52). In a study in mice, Apajalahti and colleagues found that Inulin induced community shifts that included increases of bifidobacteria and a decrease in clostridia, but the major changes were observed within previously unknown taxa (3). Therefore, although the bifidogenic effect of most prebiotic carbohydrates is clearly established, the exact effect of prebiotics on the entire community composition and structure remains an important field of study. Massively parallel sequencing of amplified 16s DNA tags via pyrosequencing now provides the means to quantify the fecal microbiota at increased depth spanning the entire microbial community at very high sensitivities. Thus, a much more detailed analysis of how prebiotics affect the microbiota can be achieved, and community wide shifts in throughout the entire phylogenetic spectrum of the bacterial population can be measured.

We recently reported that GOS, incorporated into caramel-like confections, increased the amount of bifidobacteria in 9 out of 18 healthy adults at doses above 5 g per day as assessed by specific culture, qRT-PCR, and DGGE (15). Only one other bacterial group, *Bacteroides dorei*, was detected by DGGE to become increased in one single subject, suggesting that GOS was highly specific in its stimulation of bifidobacteria. However, techniques used in our previous study were restricted in both depth and breadth, and the goal of this current study was to gain an in depth perspective of the impact of GOS in these

subjects using high throughput multiplex community sequencing of 16S rRNA tags. We discovered that GOS was remarkable for its ability to enrich specifically for bifidobacteria in human fecal samples, although it is utilized by a wide variety of bacterial inhabitants of the intestinal tract in vitro. .

MATERIALS AND METHODS

Experimental design. The details for how this study was conducted were previously described in detail (15). Briefly, caramel chews were administered to 18 healthy human volunteer during a 16 week period. The first two weeks were established as the baseline period (no chews administered), and this was followed by four sequential testing periods during which chews were administered for three weeks with GOS dosages at levels of 0.0 g, 2.5 g, 5.0 g, and 10.0 g GOS per day. A final two-week washout period (no chews) was performed at the end of the fourth testing period (weeks 15 – 16). The study was approved by the Institutional Review Board of the University of Nebraska.

Molecular characterization of the fecal microbial communities by pyrosequencing of 16S rRNA tags. Fecal samples were collected weekly and processed as described previously (15). Pyrosequencing of 16S rRNA tags was performed from fecal DNA as described by Martínez et. al (2010). Briefly, the V1-V3 region of the 16S rRNA gene was amplified by PCR from fecal DNA using

primers were modified to work with the Roche-454 Titanium kit. A mixture (4:1) of the primers B-8FM

(5'-
CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCMTGGCTCAG-
3')

and B-8FMBifido

(5'-
CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGGGTTCGATTCTGGCTCAG-
3'),

were used as the forward primers. The primer A518R

(5'-
CCATCTCATCCCTGCGTGTCTCCGACTCAGBBBBBBBBBATTACCGCGGCTG
CTGG-3') containing an 8-base barcode sequence was used as the reverse
primer. Sequences were then assigned to their respective samples via the
barcode. The 8FMBifido was used in combination with primer 8FM as 16s DNA
sequences within the genus *Bifidobacterium* are not well amplified by the latter
primer (37).

Equal amounts of the PCR products were combined, gel purified, and
sequencing was performed by the Core for Applied Genomics and Ecology
(CAGE, University of Nebraska-Lincoln) with the 454/Roche A sequencing

primer kit using a Roche Genome Sequencer GS-FLX. Using the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (<http://pyro.cme.msu.edu/>) 'Initial Process' tool, sequences were binned according to the barcode (11). Default parameters were used to remove sequences containing any ambiguous nucleotides, except for the minimum sequences length, which was set to 300 bp. BioEdit Software was used to trim the quality approved sequences to 450 bp before their submission to the sequence analyses (see below).

Sequence analyses to characterize microbial populations. Sequences obtained with pyrosequencing were analyzed using two independent approaches, a taxonomy dependent and a taxonomy independent. First, the Classifier tool of the RDP was applied (with a minimum bootstrap value of 80%) to obtain a taxonomic assignment of all sequences. The Classifier approach allowed a fast determination of the proportions of bacterial groups at different taxonomic levels (phylum, family, and genus). Second, sequences were assigned to Operational Taxonomic Units (OTUs). For this, all sequences from each subject were individually aligned using the RDP Aligner web tool, and then clustered using the RDP Complete Linkage Clustering web tool (with a maximum distance cutoff of 97%; 11). The OTU picking was done on a per subject base as the entire data from all subjects contained too many sequences for a quality alignment. Excluded from the analyses were OTUs that contained less than three sequences. Using Statistical Analysis Software (SAS) to perform ANOVA, the OTUs that were significantly affected by the dietary treatments in each

subject were identified. Representative sequences from each OTU whose abundance was significantly influenced by GOS were subjected to taxonomic classification using SeqMatch, an RDP web tool. From each statistically significant OTU identified, five random representative sequences were aligned to form consensus sequences using SeqMan Software. The consensus sequences were grouped and aligned according to phylum (Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Verrucomicrobia) together with the most closely related type strains or entry in the NCBI database using Muscle 3.6 (18). Phylogenetic trees were built by neighbor-joining with 1,000 bootstrap replicates with MEGA 4.0 Software (53). Using visual analyses and a distance matrix, OTUs were assigned as sequence clusters with >97% identity. Quantification of the OUT in each subject was performed by BLASTn analysis. For this, consensus sequences were generated for each of the OTU sequence clusters as described previously. A local nucleotide database was established through a PERL script, which combined all eighteen subjects' sequences, detected by pyrosequencing, into one database. A BLASTn algorithm was used with a 97% cutoff (min. length 300 bp) to quantify each OTU in the fecal bacterial populations within each sample. Samples that were closely related to *Bifidobacterium adolescentis* were re-analyzed with a BLASTn algorithm at a cutoff of 98% (min. length 300 bp) as clearly differentiated clusters could be identified that showed overlap with 97% algorithm. The quantification of OTUs in all subjects was verified to ensure that individual sequences were not

being assigned to different OTUs. In three occasions, seven OTUs that were initially identified as distinct had very high shared sequence similarities, and were thus merged together into three OTUs.

Determination of community diversity. Two different methods, the generation of rarefaction curves and Shannon's index, were applied to determine the diversity of the fecal microbiota using 16S rRNA sequence data. The DNA sequences of each sample were individually aligned and clustered using RDP web tools Aligner and Complete Linkage Clustering. Individual cluster files corresponding to each fecal sample were used to construct Rarefaction curves and determine the Shannon's Index.

Statistical analysis. To identify differences in fecal microbiota composition induced through dietary treatments (0.0 g, 2.5 g, 5.0 g, and 10.0 g GOS) in all eighteen subjects, one-way ANOVA tests with repeats were performed. Samples obtained during the baseline and washout periods were not included within the statistical analysis. Post hoc pair-wise comparisons were done using Tukey's method. P-values of < 0.05 were considered significant unless otherwise stated.

***In vitro* Fermentation of GOS by colonic bacteria.** A total of twenty-two anaerobic bacteria which were mainly of intestinal origin were screened for their ability to use GOS as a growth substrate. Included were *Clostridium butyricum* 23588, *Clostridium bifermentans* 23591, *Clostridium difficile* 23596, *Clostridium*

innocuum 23601, *Clostridium paraputrificum* 23600, *Clostridium perfringes* 23962, *Clostridium perfringes* 23508, *Clostridium ramosum* 23617, *Clostridium rumen* 23494, *Clostridium sporogenes* 23598, *Clostridium histolyticum* 19401, *Enterococcus faecium* 2354, *Enterococcus faecalis* 537, *Enterobacter aerogenes* 407, *Enterobacter aerogenes* 410, and *Streptococcus salivarius* 3714, *Bacteroides thetaiotaomicron* 5482, *Bacteroides distasonis* V923, *Bacteroides fragilis* 43858, *Bacteroides uniformis* BU1100, *Bacteroides fragilis* 638, and *Bacteroides ovatus* V975. Bacteria were initially propagated in Brain Heart Infusion (BHI) or Reinforced Clostridial Agar (RCA), and were transferred (2%) into a basal medium containing 5 g/L Peptone No 3 (Becton, Dickinson, and Company), 5.0 g/L Casitone (Becton, Dickinson, and Company), 0.5 g/L L-Cysteine (Sigma), 40 mL Salt Solution, 10 mL Hemin (Sigma), 900 µL Vitamin K₃ (Sigma), and 1 g/L Yeast Extract (Becton, Dickinson, and Company). In addition, cells were also inoculated into basal medium containing either 1% glucose or 1% GOS. All cultures were incubated at 37C in an anaerobic chamber (Forma Scientific, Mareitta, Ohio) containing an atmosphere of 85% Nitrogen, 10% Hydrogen, and 5% Carbon dioxide and assessed for growth by optical density measurement at 600nm in a Beckman Model 640 spectrophotometer. Each experiment was replicated in triplicate and the average optical densities were determined.

RESULTS

The effect of GOS on the fecal microbial community in human

subjects. A total of 288 fecal samples were sequenced by pyrosequencing, and 2.3 million sequences were obtained, with an average of 8,200 sequences per sample (after quality control analysis). The mean sequence length was approximately 450 bp. Subsequent identification of operational taxonomic units (OTUs) among the samples revealed an average of 2,022 OTUs per subject. To assess the effect of GOS on diversity of the fecal microbiota during the baseline, treatment, and washout periods, rarefaction curves for all eighteen subjects were generated (Figure S1). Rarefaction analyses, however, revealed that consumption of GOS did not cause significant alterations in the bacterial diversity of the fecal samples ($p < 0.0713$).

During the baseline period, the composition of the microbiota among the eighteen subjects was dominated by the phyla Firmicutes (64%) and Bacteroidetes (28%). Other phyla detected included Actinobacteria (3%), Verrucomicrobia (1%), and Proteobacteria (1%). Approximately 3% of the sequences remained unclassified. At the family level, the predominant groups were the Lachnospiraceae (31%), Ruminococcaceae (18%), Bacteroidaceae (12%), and Bifidobacteriaceae (5%). The most common genera included *Bacteroides* (12.2%), *Fecalibacterium* (7.7%), *Blautia* (7.4%), *Ruminococcus* (3.7%), *Roseburia* (2.2%), *Bifidobacterium* (1.5%), and *Dorea* (1.3%).

Sequence proportions determined by pyrosequencing were used to determine the effect of GOS on the composition of the gastrointestinal microbiota

among all 18 subjects. The groups that were significantly affected are shown in Table 1, according to phylum, family, genus (by RDP Classifier), and species (as OTUs). The control chew (no GOS) had no effect on the fecal microbiota, as the microbial populations during this period were the same as during the baseline and washout. In addition, a dose of 2.5 gram of did not induce any detectable changes within the fecal microbiota. In contrast, consumption of 5.0 g GOS led to a significant increase ($p < 0.05$) of bifidobacteria at both the family and genus level, compared to the control dose. At the species level, the abundance of only one OTU that accounted for the species , *Fecalibacterium prausnitzii*, increased significantly at this dose. A significant decrease in abundance was also observed for both the family and genus level for Bacteroidaceae ($p < 0.01$) and *Bacteroides* ($p < 0.01$), respectively, at the 5.0 g dose compared to the control.

At the 10.0 g GOS dose, taxonomy-based analysis (using Classifier) revealed differences in the proportions of several phyla. There was a significant increase in Actinobacteria compared to the control ($p < 0.001$), as well as compared to the 2.5 g dose ($p < 0.05$). This change was associated with an increase both in the family Bifidobacteriaceae and in the genus *Bifidobacterium*. The BLASTn analysis revealed that eight of the OTUs showed statistically significant differences as the GOS doses increased, with five of the OTUs being linked to known bacterial species. A significant increase was observed after the 10.0 g dose with six OTUs that account for *Bifidobacterium* species (Table 1). Three of these OTUs account for the described species *Bifidobacterium*

adolescentis, *B. longum*, and *B. catenulatum*, while three showed <97% identity to the closest type strain (Table 1, Figure 1). Interestingly, two of OTUs that did not account to any described *Bifidobacterium* species (*Bifidobacterium* spp II ($p < 0.05$), *Bifidobacterium* spp III), showed the numerically highest response to GOS (Table 1).

There were a very small number of bacterial taxa other than bifidobacteria that were influenced by GOS. Significant decreases were observed only within the family Bacteroidaceae ($p < 0.05$) and the genus *Bacteroides* ($p < 0.05$) when compared to the control dose of GOS. The OTU-based approach identified two additional taxa that differed significantly, including *Coprococcus comes* ($p < 0.05$), and *Fecalibacterium prausnitzii* ($p < 0.05$), both of which decreased after 10.0 g of GOS when compared to the control and 5.0 g, respectively. However, a significant increase in *Fecalibacterium prausnitzii* ($p < 0.05$) was observed after a dose of 5.0 g of GOS compared to the control (Table 1). There were also significant decreases that were observed within two species groups, *Fecalibacterium prausnitzii* ($p < 0.05$) when compared to the 5.0 g dose, and *Coprococcus comes* ($p < 0.05$) when compared to the control dose.

Consumption of GOS induced population shifts that were substantial but varied between subjects. The consumption of GOS at higher levels, 5.0 g and especially 10.0 g, resulted in major compositional shifts within the gastrointestinal microbiota of a subset of subjects. The most substantial alterations, numerically, were the changes observed in the abundances of the

genus *Bifidobacterium*, that increased approximately 10 fold (from 1 - 4% to 18 - 33%) in four subjects, and an about 5 fold in seven additional subjects. In contrast, there was a decrease in the abundance of *Bacteroides* in 17 subjects after the 5.0 g GOS dose, with 14 of those subjects having a further decrease after consumption of 10.0 g of GOS. The findings clearly showed that despite these substantial overall population shifts, the effect of GOS on the intestinal composition of subjects was still subject to considerable variation among individuals (Figure 2). The data revealed that there were some individuals that were essentially unaffected by GOS consumption, whereas other experienced several significant changes. The most consistent alteration detected by this analysis was the reduction in the Bacteroidetes (at the family, genus, and species levels), which occurred within all of the subjects at some point after 5.0 g of GOS was consumed (Figure 2). Other common alterations were the increase in the Actinobacteria (at the phylum, family, genus, and species levels) which was observed in sixteen of the eighteen subjects after 5.0 g and seventeen of the subjects after 10.0 g of GOS.

Temporal dynamics of microbial populations in response to GOS.

Analyses of the community profiles provided insight into how GOS influenced the population dynamics over the entire 16 week study period. All of the changes induced by GOS were reversible within one week, and no differences (Student's *t*-test, $p > 0.05$) could be detected in the proportions of the bacterial groups between the first washout sample and the baseline sample (Figure 3). The

temporal patterns of the three main phylum (Actinobacteria, Bacteroidetes, and Firmicutes) and two of the selected genera (*Bifidobacterium* and *Bacteroides*) for five representative subjects showed that these groups were stable in their temporal response to GOS. For example, levels of Actinobacteria, Bacteroidetes, and Firmicutes were remarkably stable in fecal samples at the baseline and washout periods, and their populations returned to the baseline level within one to two weeks after GOS consumption was stopped. The same observations were also made at the genus level for *Bifidobacterium* and *Bacteroides*. These taxa were significantly affected by consumption of GOS, as population dynamics were very similar throughout, indicating that these bacterial groups might be specifically targeted

In vitro growth of gastrointestinal microbiota cultures on the prebiotic GOS. The growth of twenty-two strains of bacteria, most of which originated from the human intestinal tract, in media containing GOS was compared with growth in media containing glucose (positive control; data not shown) or without an additional source of carbohydrate. In general, 6 of the 11 *Clostridium* strains could utilize GOS (Figure S3A) indicated by a higher final OD when compared to growth without carbohydrates, and in addition, three of the six strains of *Bacteroides* also grew well on GOS. Growth on GOS was not observed, however, for *Enterococcus*, *Enterobacter*, or the species included within the genus *Enterococcus* and *Streptococcus* did not result in significantly

different high levels of fermentation on GOS, when compared to the control (no GOS).

DISCUSSION

We recently reported that consumption of GOS induced bifidogenic shifts in the fecal microbial community of 18 healthy human adults (15). Daily doses of 5.0 g were generally necessary before these effects could be observed either by cultural methods, DGGE, and qRT-PCR. The results were consistent with several other studies involving human subjects consuming GOS at similar doses (7, 8, 16, 58). In addition, we also observed that when the fecal samples from each subject were analyzed individually, the bifidogenic response to GOS occurred consistently in only half of the subjects, whereas the others were consistent “non-responders” (15). However, because of the relatively low resolution of DGGE and the *Bifidobacterium*-specific primers used in qRT-PCR, we were unable to detect other changes in the microbiota that occurred as a result of GOS consumption. Therefore, all 288 samples (18 subjects at 16 weekly time points) from the previous study were used in pyrosequencing reactions to obtain individual community profiles. Our findings were entirely consistent with our previous report, but also revealed important insights regarding how GOS influences the intestinal microbiota.

Prebiotics are described, by definition, as being “selectively fermented” and able to induce changes in the gastrointestinal microbiota that are “specific” (45). Previous methods have been effective in assessing the effect of GOS consumption on the stability and diversity of the human intestinal microbiota (15, 39, 52); however, the inability to quantify the prebiotic effect beyond the major taxa has made it difficult to test this definition and to assess the effect of prebiotics at greater resolution. Results from high throughput pyrosequencing has now allowed an in depth analysis of the microbial community as a whole and has shown for the first time that GOS induces changes that are remarkably selective. Indeed, the only bacteria that consistently increased in abundance in response to GOS feeding were bifidobacteria. Moreover, this increase in bifidobacteria abundance, to greater than 20% in some individuals, was generally at the expense of only one group of bacteria, namely the genus *Bacteroides*. Although abundance of *Bifidobacterium* increased in some individuals without a commensurate decrease in *Bacteroides* (and vice versa), in general, bifidobacteria increased and bacteroides decreased (Table 1, Figure 2) during GOS consumption. This data clearly shows that GOS, as a prebiotic, is highly specific toward the Actinobacteria phylum, in particular bifidobacteria (Figure 1B).

In this study, we observed not only changes at phylum, family, and genus level, but perhaps more importantly, also at the species level (Figure 2A and B). The most consistent response, at the 10.0 g treatment, was an increase of *Bifidobacterium adolescentis* that was detected in eight out of the eighteen

subjects. Interestingly, this was the same species that we previously identified from DGGE analyses and that Tannock et al. (2004) also detected in response to GOS consumption. Although *Bifidobacterium* are generally associated with humans and animals, *B. adolescentis*, in particular, is common in children and dominant in adults (4, 5, 6, 40). The abundance of two other *Bifidobacterium* species also increased at the 10 g GOS dose, *Bifidobacterium longum* and *Bifidobacterium catenulatum*, as well as three unidentifiable *Bifidobacterium* spp (Table 1; Figure 1A and B). This data suggests that while GOS is highly specific toward the genus *Bifidobacterium*, the ability to ferment GOS appears to extend to only a few species.

As we noted previously, the response to GOS consumption is subject to considerable individual variation (38), an observation confirmed by the pyrosequencing data. Of the 54 OTUs that were identified in individual subjects, 46 did not reach significance when all of the subjects were included in the analysis. In addition, none of the taxa that were significantly affected by GOS showed a response in all eighteen subjects. Of the eight OTUs that were identified as significant, only two were closely identified with *B. adolescentis*. Due to the high percentage of shared sequences, these two OTUs were re-analyzed with a 98% similarity score, as well as a chimera test. They were subsequently classified as separate OTUs, confirming that this species was most commonly increased (seen in eight subjects) with consumption of GOS (Figure 1A).

The highly individual response to GOS may occur for one of several possible reasons. First, the presence or absence of specific strains, capable of metabolizing GOS, would appear to be the major determinant, as few OTUs are completely conserved among humans (54, 57). Thus, the presence of specific GOS-metabolizing strains would confer responder status on that individual, other individuals in which GOS strains are absent would be non-responders. Other factors could also account for these results, including host specific environmental constraints that would restrict the ability of the bacterial group to increase in numbers even if a suitable substrate is provided (38). In addition, host digestive enzymes may be secreted that affect the amount of GOS that withstands digestion.

The ability of GOS to support growth of a range of colonic bacteria, as we observed in this study (Figure S3A), would appear to be inconsistent with the very definition of a prebiotic. The ability of species of *Clostridium*, *Bacteroides* and *Streptococcus* to utilize GOS in pure culture, has previously been reported, as well Rycroft et al. (2001). Clearly, however, the substrate preferences and competitive forces that exist in the gastrointestinal environment are quite different from pure monoculture environments. Although the abundance of Actinobacteria was much lower than Firmicutes or Bacteroides during baseline and control periods, only the former group was stimulated by GOS, despite the apparent ability of the latter groups to grow on GOS. Thus, it would appear that a competitive environment is necessary to demonstrate a prebiotic effect.

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Table 1. Abundance of bacterial taxa that were impacted by GOS consumption in fecal samples of eighteen human subjects as determined by pyrosequencing of 16S rRNA tags.

Proportion of bacterial taxa expressed in percentage (Mean \pm SD)							
Phylum	Baseline ¹	0.0 g ²	2.5 g ²	5.0 g ²	10.0 g ²	Washout ¹	P value ³
Actinobacteria	2.52 \pm 2.34	2.58 \pm 3.59	3.69 \pm 4.33	5.39 \pm 6.11	7.19 \pm 8.88	2.09 \pm 2.51	< 0.0001
Family							
Bifidobacteriaceae	1.87 \pm 2.28	2.03 \pm 3.59	2.99 \pm 3.72	5.11 \pm 5.77 *	7.36 \pm 8.68	1.49 \pm 2.36	< 0.0001
Bacteroidaceae	12.22 \pm 7.43	15.03 \pm 10.66	13.29 \pm 9.24	11.20 \pm 9.11 **	11.66 \pm 9.22 *	13.69 \pm 8.27	0.003
Genus							
<i>Bifidobacterium</i>	1.54 \pm 1.95	1.68 \pm 2.97	2.55 \pm 3.25	4.32 \pm 5.03 *	6.24 \pm 7.71 ***§§	1.26 \pm 2.04	< 0.0001
<i>Bacteroides</i>	12.22 \pm 7.43	15.03 \pm 10.66	13.29 \pm 9.24	11.20 \pm 9.11 **	11.66 \pm 9.22 *	13.69 \pm 8.27	< 0.0001
Species (OTUs)							
<i>B. adolescentis</i>	0.37 \pm 0.56	0.34 \pm 0.89	0.46 \pm 0.86	0.85 \pm 1.09	1.03 \pm 1.55 *	0.21 \pm 0.48	0.0101
<i>Bifidobacterium spp I</i>	0.15 \pm 0.36	0.18 \pm 0.33	0.25 \pm 0.55	0.52 \pm 1.13	0.77 \pm 1.41 *§	0.12 \pm 0.25	< 0.0001
<i>Bifidobacterium spp II</i>	0.46 \pm 0.94	0.60 \pm 1.53	0.76 \pm 1.72	1.41 \pm 2.38	2.00 \pm 3.45 *§	0.22 \pm 0.45	< 0.0001
<i>Bifidobacterium spp. III</i>	0.62 \pm 1.21	0.78 \pm 2.19	0.98 \pm 2.02	1.82 \pm 3.30	2.50 \pm 4.55 *§	0.40 \pm 0.92	0.0088
<i>B. longum</i>	0.09 \pm 0.23	0.09 \pm 0.23	0.12 \pm 0.32	0.22 \pm 0.50	0.33 \pm 0.85 *	0.15 \pm 0.38	0.0232
<i>B. catenulatum</i>	0.15 \pm 0.34	0.27 \pm 0.88	0.56 \pm 1.38	0.51 \pm 1.16	0.91 \pm 2.08 **	0.28 \pm 0.78	0.0105
<i>F. prausnitzii</i>	3.52 \pm 2.71	3.21 \pm 2.26	3.71 \pm 2.67	4.37 \pm 3.67 *	3.16 \pm 1.82 †	3.42 \pm 2.28	< 0.0001
<i>Coprococcus comes</i>	2.90 \pm 2.04	2.40 \pm 1.75	2.12 \pm 1.24	1.99 \pm 1.55	1.78 \pm 1.11 *	2.15 \pm 1.30	< 0.0001

¹ Bacteria populations are averages of the two time points of the baseline period and the two time points of the washout period.

² Bacteria populations are averages of all three time points of the feeding periods

³ Bacterial populations during the dietary treatments were compared to each other with repeated measures ANOVA and Tukey's post hoc test

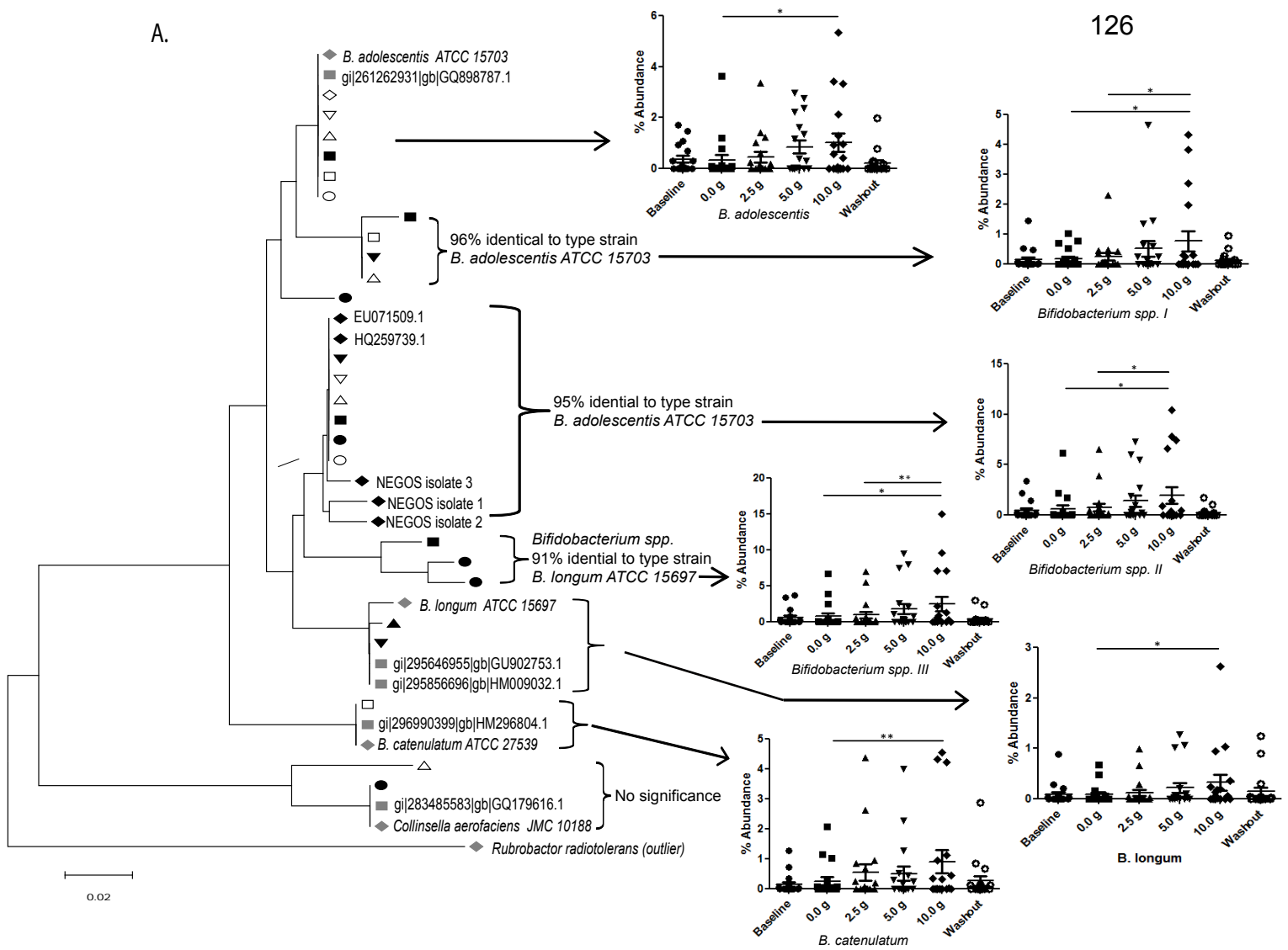
Significantly different to 0.0 g: *(p < 0.05), ***(p < 0.001)

Significantly different to 2.5 g: §(p < 0.05), §§(p < 0.01)

Significantly different to 5.0 g: †(p < 0.05)

Figure 1. Characterization of the fecal microbiota in eighteen subjects that consumed increasing doses of GOS by multiplex pyrosequencing of 16S rRNA tags. A phylogenetic tree that encompasses the phylum **(A)** Actinobacteria is shown. The tree contains representative sequences of all OTUs detected to be impacted by GOS in individual subjects together with sequences of related entries in the database (which included both type strains of known species and sequences from molecular studies of human fecal samples). Sequences were aligned in Muscle 3.6 and the trees were built using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA 4.0. Open black and closed black symbols were used to label sequences from individual subjects. OTUs that were not significantly affected in all eighteen subjects were labeled as 'No significance.' The graphs next to the trees show the abundance of OTUs and bacterial groups that were significantly altered during the dosages (0.0 g, 2.5 g, 5.0 g, and 10.0 g). A graph **(B)** that incorporates all of the *Bifidobacterium* species altered during consumption of GOS, for all eighteen subjects, is also shown. These graphs show mean proportions of the three individual samples taken during the treatment periods for each subject. Baseline and washout refer to samples taken in periods where no GOS was consumed. Repeated measures ANOVA in combination with a Tukey's post-hoc test were performed to identify differences between treatment groups, and the baseline/washout periods were not included in the statistic analysis. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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B.

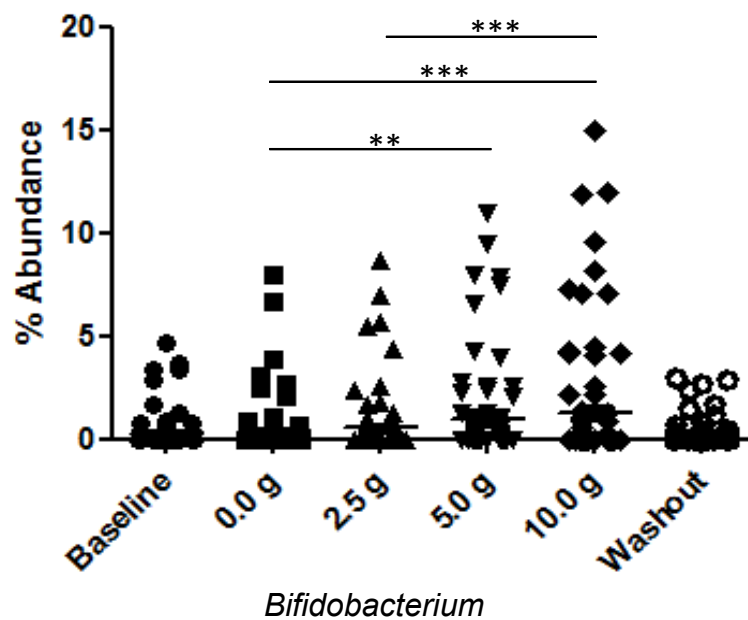


Figure 2. Bubble plots showing differences in the proportions of bacterial taxa as a percentage of the whole bacteria population detected during consumption of 5.0 g **(A)** and 10.0 g **(B)** when compared to the control period. The size of the bubbles is representative of the percent difference. Black ovals represent increases in proportions induced through GOS consumption, and white ovals represent a decrease.

Figure 2.

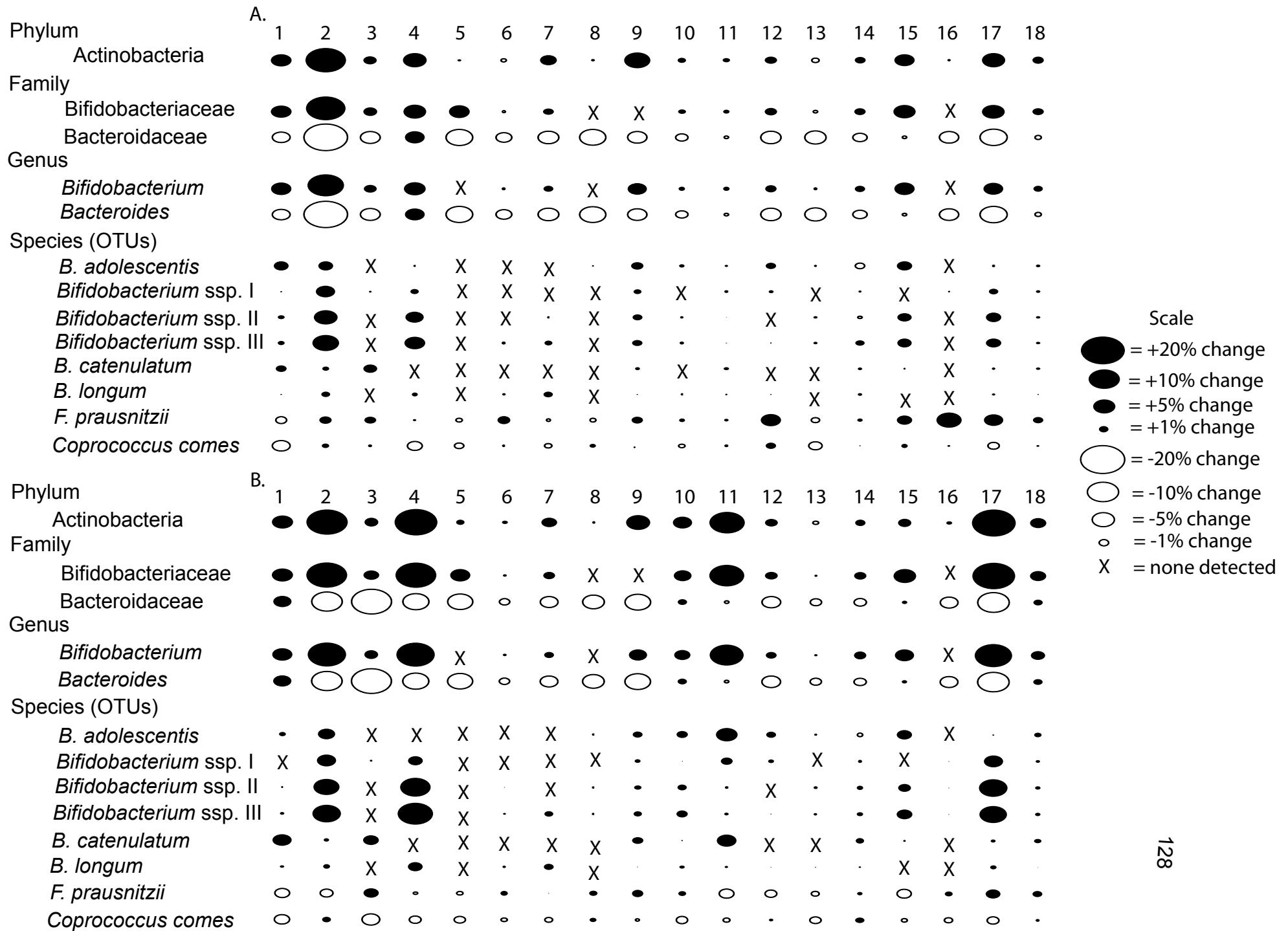


Figure 3. Temporal dynamics of the human fecal microbiota in response to the consumption of increasing doses of GOS shown in five human subjects. Graphs on the left show proportions of the three main phyla and two genera (*Bifidobacterium* and *Bacteroides*) altered for subjects considered ‘responders’, which graphs on the right show proportions of the same three main phyla and two genera altered for subjects considered ‘non-responders’.

Figure 3.

Responders

Non-Responders

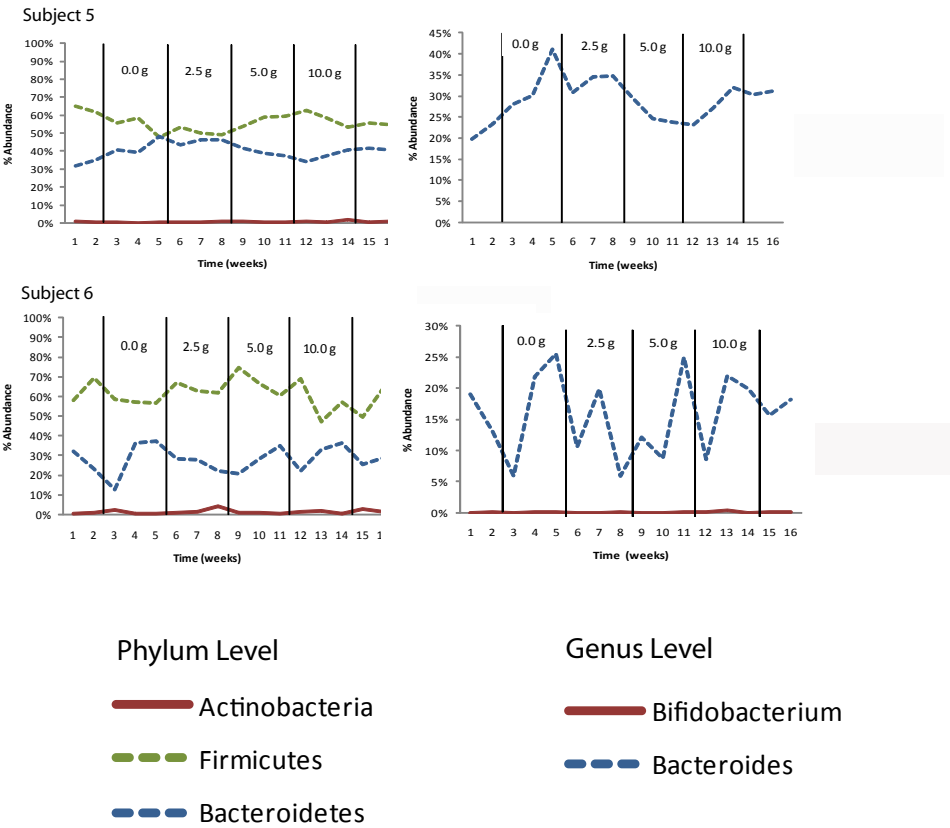
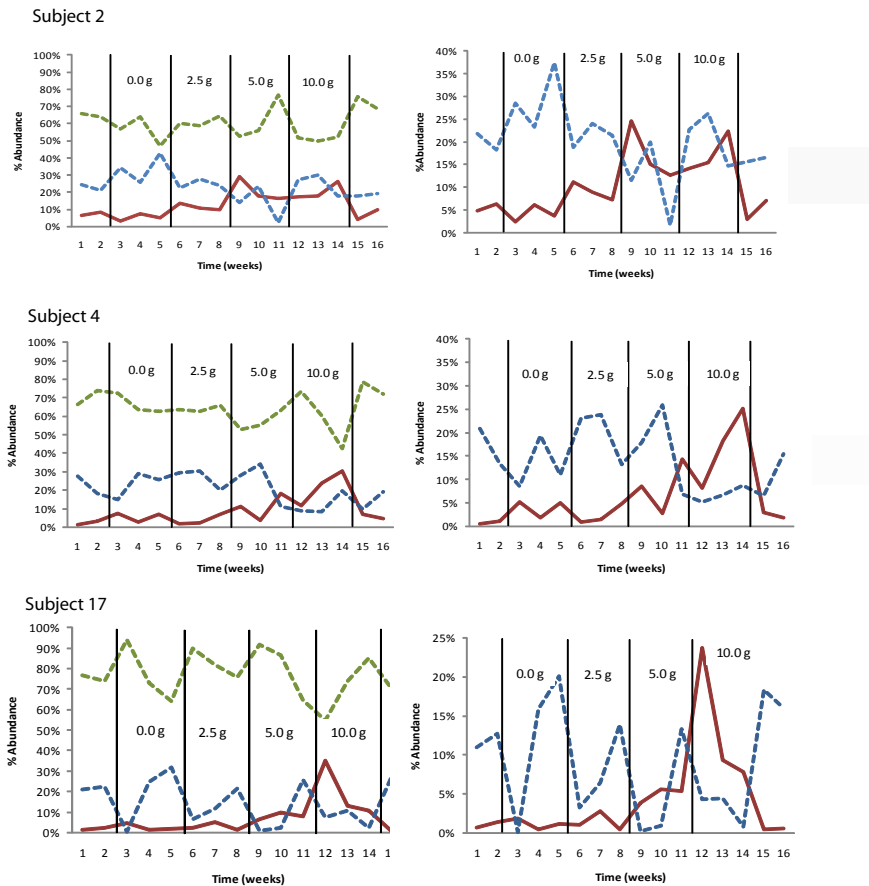


Figure S1. Diversity of species richness of the fecal microbiota in eighteen human subjects that consumed doses of 0.0 g, 2.5 g, 5.0 g, and 10.0 g of GOS. Rarefaction curves showing the amount of OTUs in all individual fecal samples taken from eighteen subjects.

Figure S1.

Number of OTUs

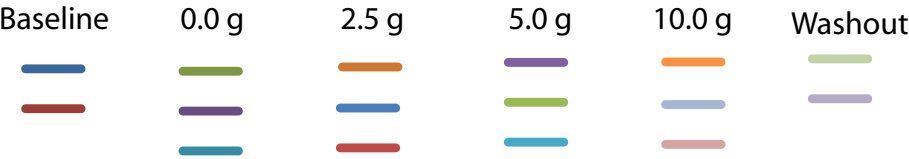
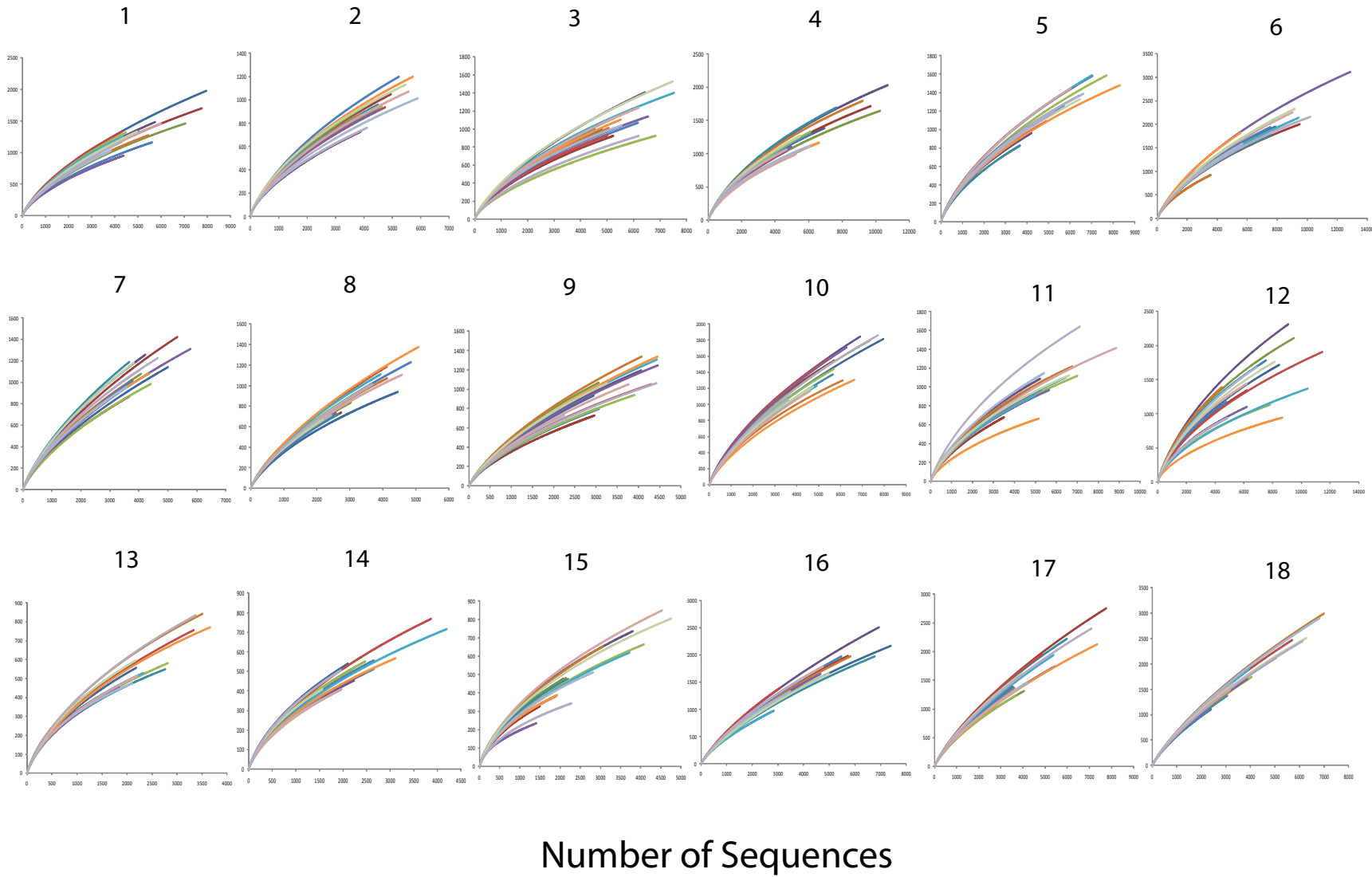
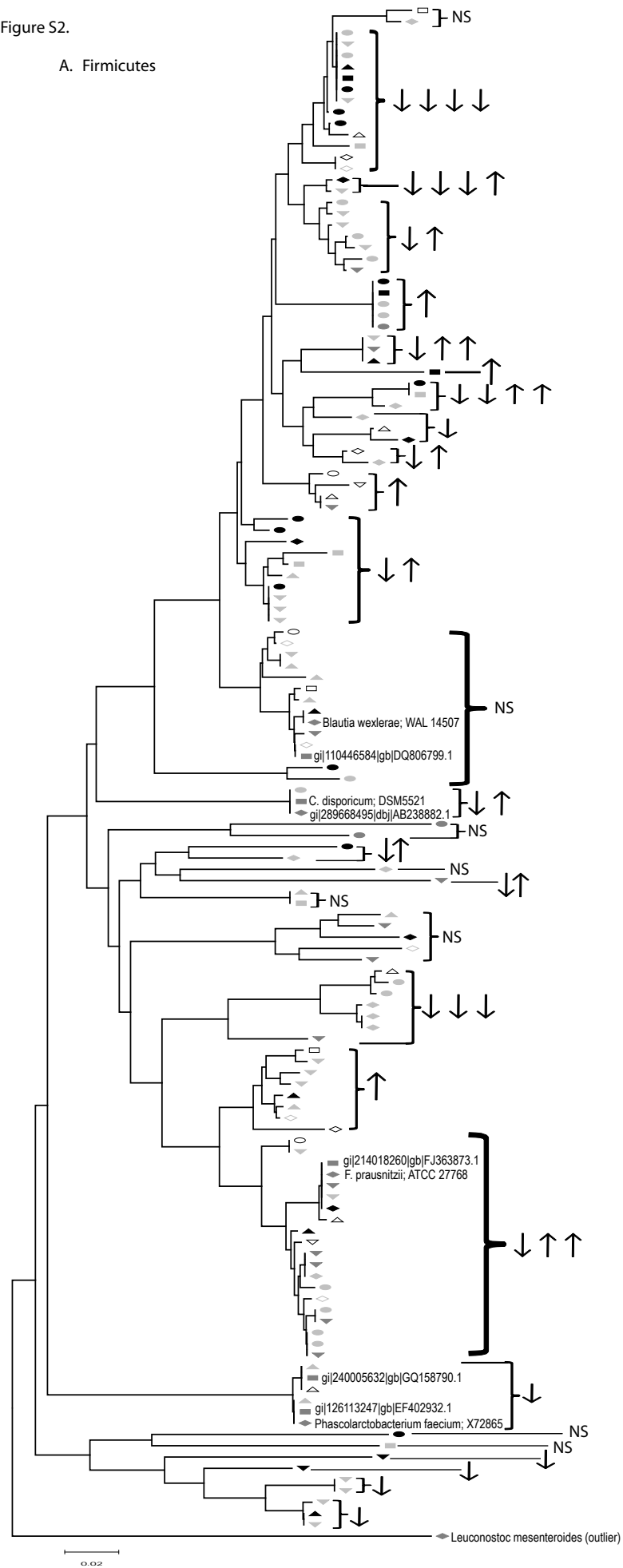


Figure S2. Characterization of the fecal microbiota in eighteen subjects that consumed increasing doses of GOS by multiplex pyrosequencing of 16S rRNA tags. Phylogenetic trees that encompass the phyla **(A)** Firmicutes and **(B)** Bacteroidetes are shown. The trees contain representative sequences of all OTUs detected to be impacted by GOS in individual subjects together with sequences of related entries in the database (which included both type strains of known species and sequences from molecular studies of human fecal samples). Sequences were aligned in Muscle 3.6 and the trees were built using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA 4.0. Open black and closed black symbols were used to label sequences from individual subjects. OTUs that were not significantly affected in all eighteen subjects were labeled as 'No significance.' Arrows to the right of each cluster indicate the number of subjects that showed statistical significance after ANOVA analysis. The direction of the arrow indicates either a significant increase (↑) or significant decrease (↓) for each subject showing significance for that particular OTU cluster.

Figure S2.



B. Bacteroidetes

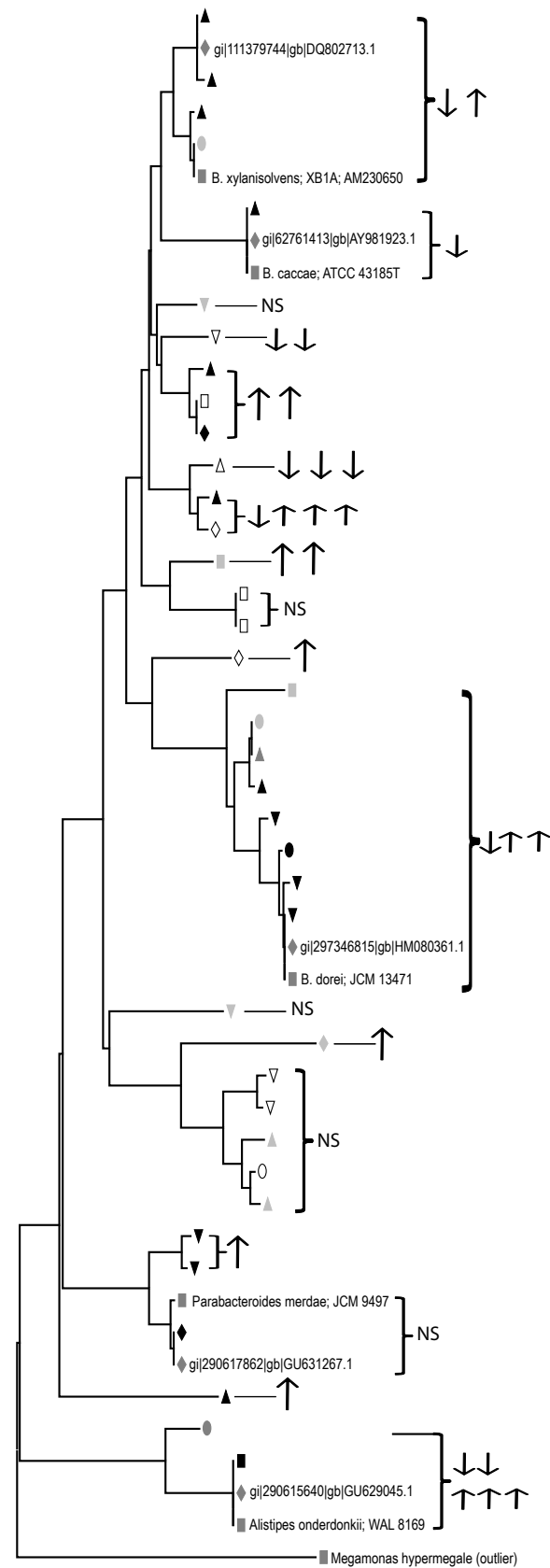
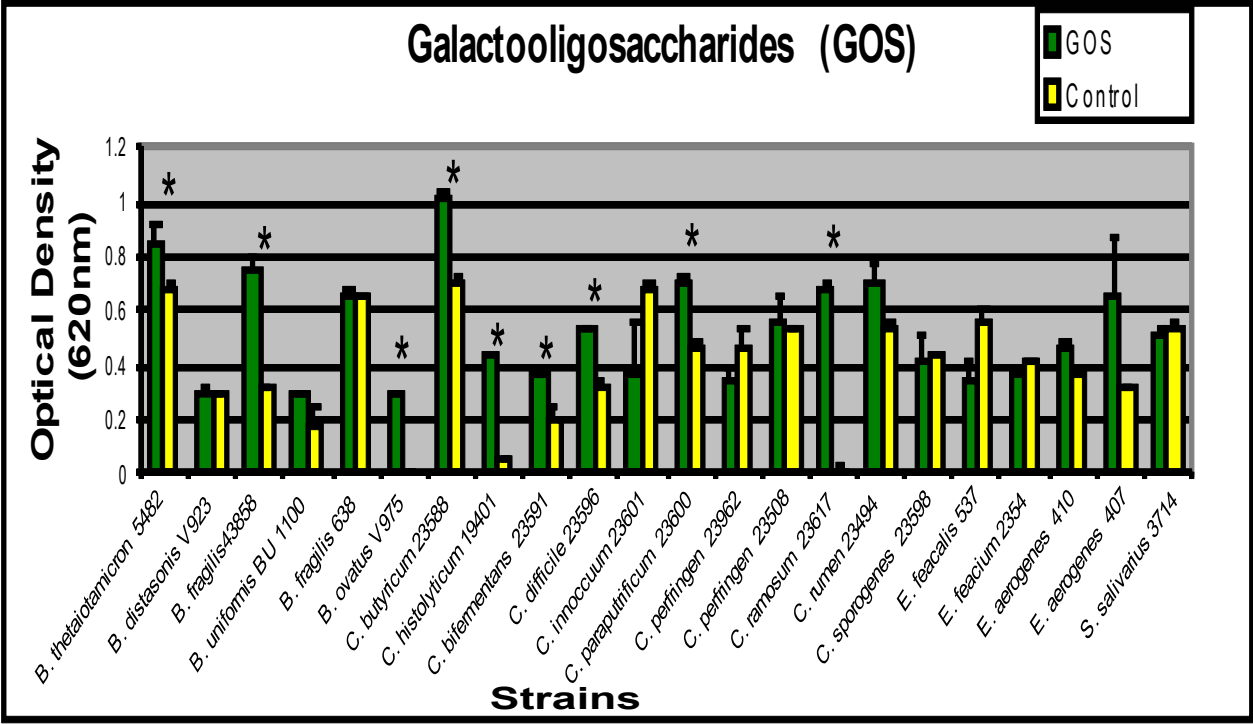


Figure S3. Twenty-two anaerobic bacteria of human gastrointestinal origin were screened *in vitro* to determine their ability to utilize GOS. Optical density for each of the strains is shown **(A)**, with significant differences determined by students T-test and indicated by (* $p < 0.05$). Each of the strains used in the study **(B)** were obtained from the USDA.

Figure S3.

A.



B.

- Bacterial Strains
- Bacteroides thetaiotamicron* 5482
 - Bacteroides distasonis* V923
 - Bacteroides fragilis* 43858
 - Bacteroides uniformis* BU 1100
 - Bacteroides fragilis* 638
 - Bacteroides ovatus* V975
 - Clostridium butyricum* 23588
 - Clostridium histolyticum* 19401
 - Clostridium bifementans* 23591
 - Clostridium difficile* 23596
 - Clostridium innocuum* 23601
 - Clostridium paraputrificum* 23600
 - Clostridium perfringens* 23962
 - Clostridium perfringens* 23508
 - Clostridium ramosum* 23617
 - Clostridium rumen* 23494
 - Clostridium sporogenes* 23598
 - Enterococcus faecalis* 537
 - Enterococcus faecium* 2354
 - Enterobacter aerogenes* 410
 - Enterobacter aerogenes* 407
 - Streptococcus salivarius* 3714
- * All bacterial strains were obtained from the USDA

Chapter 4

Conclusion

In this research, we established that consumption of the prebiotic GOS induced changes on the composition of the human gastrointestinal microbiota. Evidence for this conclusion was exhibited using both cultural and molecular methods to enumerate and characterize the shift in selected bacterial populations. Specifically, selective plating methods were used to enumerate bifidobacteria, *Bacteroides*, enterobacteria, Enterococci, lactobacilli, and total anaerobes. Culture-independent methods included denaturing gradient gel electrophoresis (DGGE), quantitative real-time PCR (qRT-PCR), and high throughput multiplex community sequencing of 16S rRNA tags. We hypothesized that after a particular dosage level of GOS, a bifidogenic response would occur. Also, we anticipated that GOS would have a community wide affect outside of the known bifidobacteria population. Described below are the major findings of this research.

- A dose response relationship necessary to elicit a bifidogenic effect in a majority of subjects was seen after a 5 g dose of high purity GOS.
- Even when GOS was administered for many weeks and at high doses, there were still some individuals for whom a bifidogenic response did not occur; this results supports the concept that some individuals are responders whereas other are non-responders.
- The population shifts caused by consumption of 10 g of GOS were numerically substantial, leading to a ten-fold increase in bifidobacteria in

four subjects, enriching them to 18-33% of the fecal microbial community, and a five-fold increase in seven additional subjects.

- The increase in bifidobacteria abundance was generally at the expense of only one group of bacteria, namely the genus *Bacteroides*.
- GOS is utilized by a wide variety of bacterial inhabitants of the intestinal tract when studied in vitro, but is remarkable for its ability to enrich very specifically for bifidobacteria in vivo.